MODULATION OF THE PLASMA MEMBRANE DOMAIN STRUCTURE OF HUMAN NEUTROPHILS

by

Jamal Talal Stie

A dissertation submitted in partial fulfillment
of the requirements for the degree of

of

Doctor of Philosophy

in

Microbiology

MONTANA STATE UNIVERSITY
Bozeman, Montana

July/2006

APPROVAL

of a dissertation submitted by

Jamal Talal Stie

This dissertation has been read by each member of the dissertation committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the Division of Graduate Education.

Approved by Committee Chair

Algirdas J. Jesaitis

Approved for the Department of Microbiology

Tim Ford

Approved for the Division of Graduate Education

Joseph Fedock

STATEMENT OF PERMISSION TO USE

In presenting this dissertation in partial fulfillment of the requirements for a doctoral degree at Montana State University, I agree that the Library shall make it available to borrowers under rules of the Library. I further agree that copying of this dissertation is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for extensive copying or reproduction of this dissertation should be referred to ProQuest Information and Learning, 300 North Zeeb Road, Ann Arbor, Michigan 48106, to whom I have granted "the exclusive right to reproduce and distribute my dissertation in and from microform along with the non-exclusive right to reproduce and distribute my abstract in any format in whole or in part."

Jamal Talal Stie

July, 2006

TABLE OF CONTENTS

TABLE OF CONTENTS-Continued

TABLE OF CONTENTS-Continued

TABLE OF CONTENTS-Continued

TABLE OF CONTENTS-Continued

LIST OF TABLES

LIST OF TABLES-Continued

LIST OF FIGURES

LIST OF FIGURES-Continued

LIST OF FIGURES-Continued

LIST OF FIGURES-Continued

ABSTRACT

Eukaryotic cell plasma membranes form an interface between cells and their environment and function to detect and interpret environmental cues. The work described in this dissertation examines the changes that occur in membrane structure during plasma membrane function in human neutrophils and a fungal opportunist. The body of this work examines how circulating neutrophils can remain functionally inactive in the presence of perturbing influences inherent in the blood circulation, and yet rapidly activate upon exposure to proinflammatory agents. It is hypothesized that the regulated modulation of plasma membrane domain structure determines the activation of blood-leukocytes, *in vivo*. Experimentation is based the isolation of blood-neutrophils in either nonactivated or activated (primed) cellular states using dextran- or gelatin-based preparative methods, respectively. Analysis of plasma membrane cortical components actin, fodrin, ezrin, CD45 and CD43 by sucrose density sedimentation, flow cytometry and indirect immunofluorescence microscopy indicated significant differences in the plasma membrane structure of both neutrophil populations. In nonactivated neutrophils, cortical actin and fodrin were cytosolic, thus indicating the absence of cortical structure in this population. However, cortical actin and fodrin were membrane-associated in activated neutrophils showing the existence of a cortex. Fodrin, actin, ezrin and their respective anchors, CD45 and CD43 did not codistribute with the plasma membrane marker, alkaline phosphatase, in sucrose density gradients made with primed neutrophils. These latter results suggested the lateral compartmentalization of the plasma membrane cortex into compositionally distinct surface domains. Additional studies were performed to examine the surface-association of hCap, a soluble microbicidal component of neutrophil specific granules. Results indicated association of hCap with primed and degranulated but not nonactivated plasma membranes. This interaction was resistant to 1M salt but labile to 10 mM NaOH, indicating a high affinity association. In support of this, hCap also co-partitioned with detergent in Triton X-114 phase experiments. In separate studies, marked alterations in the plasma membrane lipid metabolism of isolates from *Candida glabrata* are correlated with an ability to survive and grow *in vivo*. Altogether, this work provides insight into structure-function relationships at the plasma membrane level.

CHAPTER 1

INTRODUCTION

Overview of Formyl Peptide Receptor Signaling and Compartmentalization in Human Neutrophils

The formyl peptide receptor (FPR) is a heptahelical transmembrane protein that associates with membrane-bound heterotrimeric G protein (Bommakanti et al., 1994). Agonist-bound FPR catalyze the activation of G protein subunits which in turn initiate multiple signaling cascades that drive the classical effector responses characteristic of neutrophils. As one of several classes of chemoattractant receptors that are constitutively expressed in neutrophils, the FPR drives directional movement along chemoattractant gradients, enabling the arrival of defense cells into areas of infection (Miettinen et al., 1997).

In N-formyl Met-Leu-Phe (fMLF)-stimulated neutrophils, activated FPR are localized regionally at the leading edge of the protruding membrane during chemotaxis (Servant et al., 2000). The physical and biochemical events that underlie the dynamic properties of neutrophil function derive from protein-protein as well as protein-lipid interactions. One intriguing but speculative aspect of protein-lipid signaling pathways concerns the cooperative, or synergistic, nature of interaction that transpires. According to a recently proposed model, leukocyte chemotaxis results from a positive feedback interaction between cytosolic mediators and phospholipids species present in the pseudopodial membrane (Parent and Devreotes, 1999). During chemotaxis, this cooperative interaction results in a compartmentalized, asymmetric signal amplification

proximal to the inner leaflet of the cell's leading edge membrane, thus facilitating directional movement (Parent and Devreotes, 1999;Servant et al., 2000). In support of this proposal, investigators have previously noted the absence of receptor clustering during either fMLF- or C5a-stimulated neutrophil chemotaxis (Servant et al., 1999;Servant et al., 2000). Instead, signaling gradients are generated downstream of activated receptors by the local recruitment and subsequent marked accumulation of cytosolic signaling proteins within membrane regions proximal to the activated receptors (Servant et al., 2000). The spatial and temporal events that contribute to this polarization both underscore the existence of distinct topological regions in the phospholipid make-up of plasma membranes as well as a unique capacity of submembranous cytoskeletal structure to compartmentalize signaling events and associated mechanical responses.

The FPR expressed in human neutrophil plasma membranes exist in topological regions that are compositionally and structurally distinct. Compositionally, the neutrophil plasma membrane consists of 10% phosphatidylinositol (Dupou et al., 1988). This phospholipid class is of significance because its metabolites serve as substrates for G-protein-activated, membrane-bound phospholipases. Furthermore, phosphoinositides have diverse effector properties that (i) facilitate the docking, fusion, and budding of cytoplasmic vesicles to/from plasma membranes (Cockcroft, 1996); and (ii) affect structural reorganization of the membrane-associated cytoskeleton (also known as the membrane cortex or membrane skeleton) (Schmidt and Hall, 1998).

The membrane skeleton contributes to the topological organization of membrane components by (i) subdividing the plasma membrane into structural compartments and by

(ii) regulating the free diffusion of membrane receptor proteins and/or their associated downstream signaling proteins. A primary consequence of membrane-cortical interactions is the formation of a lattice structure that defines cell shape, stabilizes cell structure, and both organizes the topology of integral and peripheral proteins and, in many cases, regulates their activity. Many of these interactions involve phosphoinositides, which can affect membrane skeletal organization, cell shape, and plasma membrane function by modulating, either directly or indirectly, membrane skeleton-plasma membrane interactions. Thus, the lateral transmembrane organization of plasma membrane phospholipids, submembranous cytoskeletal structure, and the physical/biochemical properties of membrane receptors work cooperatively to optimize cellular responses to external stimuli (Melamed et al., 1991;Melamed and Gelfand, 1999).

Transient changes in membrane skeletal organization are a major consequence of membrane receptor activation. A central downstream event following membrane receptor activation is the induction of phosphoinositide metabolism, including the local synthesis of phosphoinositides and their selective enzymatic degradation. Such receptor-mediated events culminate in phosphoinositide-directed, transient changes in membrane skeletal organization that can potentially coordinate the assembly of G-protein activated signaling pathways at the cell surface with precise changes in the compartmentalization of plasma membranes. Notably, various phosphoinositide species are differentially enriched in functional microdomains of plasma membrane bound FPR (A. J. Jesaitis, unpublished data), thus suggesting a molecular basis for FPR compartmentalization in fMLF-activated

and desensitized neutrophils. Membrane ‘compartmentalization’ or ‘microdomains’ refers to the structural partitioning of surface membranes into divisible sectors that are differentially enriched in various skeletal proteins, transmembrane and peripheral proteins, and/or membrane lipid classes. The relative molecular composition and dynamic properties of FPR-enriched compartments in human neutrophil plasma membranes are some of the major points examined in this dissertation.

Another common mechanism by which plasma membrane phosphatidylinositides and/or the plasma membrane-associated membrane skeleton can alter membrane microdomain structure is by regulating intermembrane fusion events. The antimicrobial activity of human neutrophils is governed by the regulated fusion of distinct granule subpopulations and secretory vesicles with the plasma membrane. The initial, selective exocytosis of secretory vesicles occurs early during neutrophil activation and is essential for the conversion of quiescent, circulating cells to adherent effector cells capable of extravasation, cellular chemotaxis, superoxide generation, and phagocytosis. *Exactly how this initial fusion event changes the effector capacity of neutrophil plasma membranes is only partially known.* For example, although it is established that upregulated secretory vesicles supply the plasma membrane with protein and lipid components necessary for functional conversion, a regulatory or organizational context for the activity of such components following their delivery to plasma membranes is relatively unexplored. Even so, the rapid transition of neutrophils from nonactivated to activated cellular states after secretory vesicle-plasma membrane fusion suggests an organizational context for the delivered secretory vesicle components in the surface membrane and, therefore, the

regulation of their activity. Elucidating the precise changes in plasma membrane structure that occur in response to secretory vesicle exocytosis could therefore provide insight into how neutrophils initially activate in response to extracellular cues. The potential role of plasma membrane remodeling in response to secretory vesicle exocytosis and the functional activation of neutrophils is an important, recurring aspect neutrophil physiology examined in this dissertation.

Mammalian cell plasma membrane phosphoinositide metabolism and membrane skeletal organization are commonly characterized in relation to more prevalent class of microdomains referred to as membrane rafts. Rafts are structures composed of various subclasses of saturated phospholipid and sphingolipid that laterally interact in the plane of the membrane to form freely diffusable, submicron-to-micron clusters. Membrane rafts may comprise from 10% to 80% of the membrane surface area, depending on cell type, and function to coordinately regulate the organization and activity of plasma membrane receptors under diverse *in vitro* conditions of cellular activation by cognate ligands (Simons and Ikonen, 1997). Rafts dynamically form in the plasma membranes of activated leukocytes, with half-lives from milliseconds to minutes, and have been implicated as essential factors governing the specificity and kinetics of signal transfer at the plasma membrane level.

It has been proposed that the regional subcellular organization of membrane receptor and/or downstream signaling components is necessary for the efficient and specific transduction of receptor-mediated signals (Schreiber et al., 2000). The submembranous cytoskeletal aspect of plasma membrane structure may contribute to the

dynamic formation and/or reorganization of membrane rafts by selectively interacting with and compartmentalizing cytosolic signaling pathway components in proximity with the cytoplasmic aspect of various classes of raft-localized integral plasma membrane receptors (Davy et al., 2000a). FPR organization and activity is differentially regulated in human neutrophil plasma membranes by primary structural components of the membrane skeleton, actin and fodrin, in response to various experimental contexts cellular stimulation with fMLF. Although membrane rafts have also been implicated in FPR organization during fMLF-facilitated neutrophil chemotaxis, a direct link between receptor activity and receptor-raft localization has not yet been established.

Overview of Dissertation

Cell-specific changes in plasma membrane organization coordinate a variety of functionally diverse processes in mammalian cells, including the formation, stabilization, and cellular function of (i) absorptive microvilli in mucosal epithelia (Danielsen and Hansen, 2006), (ii) tissue architecture (Shen and Turner, 2005), and (iii) inducible chemosensory structures assumed by neuronal cells and leukocytes (Parent, 2004;Twiss and van Minnen, 2006;Wen and Zheng, 2006). The conclusions from studies aimed at elucidating the functional basis of plasma membrane organization of any given cell type can, by extension, provide a basic framework for understanding related phenomena in other, diverse mammalian cell types. The major goal of the research presented in this dissertation is to further elucidate the functional organization, composition, structure and dynamics of the mammalian cell plasma membrane and its primary structural component,

the plasma membrane-associated cytoskeleton and thereby add to current understanding of mammalian cell plasma membrane structure and its role in leukocyte activation.

A detailed overview of the principal factors governing plasma membrane organization in mammalian cells is provided in the Background Section of this research. This section first describes the structure and composition of the mammalian cell plasma membrane in terms of its lipid and underlying cortical cytoskeletal components. Subsequently, mechanisms by which membrane lipids and proteins affect the compartmentalization and functional organization of plasma membranes are discussed. The impact of such mechanisms on the functional activation of leukocytes, particularly human neutrophils, is then presented. In order to evaluate the described and potential roles of microdomain formation in FPR signaling and neutrophil function, an overview of neutrophil physiology and the physiological properties that govern human neutrophil activation is also presented.

The following two chapters, including Chapters 2 and 3, document dynamic, large-scale changes that occur in the cellular and/or membrane physiology of human neutrophils at precise, phenotypically well-defined stages of cellular activation. Chapter 2 examines the foremost physiological event in neutrophil activation, secretory vesicle exocytosis, and the nature of its integration into surface membranes. A model system is developed to address these issues and thus allow characterization of neutrophil populations before and after secretory vesicle exocytosis. This model system consists of two neutrophil populations purified from whole blood using two different methodologies that promote either the preservation or selective loss of intact secretory vesicles within

purified cellular populations. The differences in secretory vesicle content of purified neutrophil populations are due to the ability of the blood-neutrophil isolation strategies employed to reproducibly and uniformly modulate the cellular activation state of neutrophils during the purification process, such that resting (nonactivated) neutrophil populations retain their secretory vesicles while activated (primed) neutrophil populations completely lack this organelle.

One major problem addressed in this chapter is the significant, variable retention of intact secretory vesicles within primed neutrophil populations. This unique ability also obscures the primary basis of phenotypic distinction between neutrophil populations. In particular, the incomplete surface translocation of secretory vesicles in activated neutrophil populations promotes unacceptable variation in the subcellular fractionation of marker activities used to evaluate and characterize plasma membrane structure. Thus, different methods of preparation yield cells containing different amounts of secretory vesicles. These differences preclude a detailed comparative study between nonactivated and primed populations and also prevent the comparison of results obtained with related data from different laboratories. This chapter, therefore, additionally examines (i) procedural considerations of blood-neutrophil isolation that modulate the extent of secretory vesicle upregulation in purified populations; and describes (ii) a modified protocol for blood-neutrophil preparation that reproducibly and uniformly promotes the complete loss of secretory vesicles in isolated neutrophil populations.

A major concern associated with secretory vesicle exocytosis in activated human neutrophils is the potential for convoluted, possibly artifactual, alterations in bilayer

structure that arise from incomplete secretory vesicle-plasma membrane fusion events. This variable integration of cell-surface upregulated secretory vesicles is associated with their unique capacity to undergo a compound homotypic fusion process prior to their fusion with plasma membranes by a process similar to the compound exocytic behavior of eosinophilic granulocytes and other related lineages (Kobayashi and Robinson, 1991;Jiang et al., 1998). The work described in Chapter 2 examines this issue and demonstrates that the membrane integrity of activated neutrophil populations remains uncompromised following selective upregulation of secretory vesicles and, furthermore, shows that secretory vesicle membranes are uniformly integrated into existing plasma membrane structure.

The experimental model system described in Chapter 2 is employed by work presented in Chapter 3 as a basis for demonstrating large-scale organizational and compositional changes in plasma membrane structure that occur in response to neutrophil activation and the selective exocytosis of secretory vesicles. In this study, neutrophil populations occupying resting and primed cellular states are purified from whole blood using the different preparative methods described in Chapter 2. The work described in this chapter begins with functional studies that confirm the cellular activation states occupied by the resting and activated neutrophil populations designated in Chapter 2, which identified resting and activated neutrophil populations on the basis of the respective retention and complete absence of intact secretory vesicles within purified neutrophil populations. However, this inference, although well founded on previously published functional studies equating secretory vesicle loss with neutrophil priming, may

be considered questionable for several reasons. First, the *in vitro* conditions employed for cellular stimulation differ significantly between the functional studies cited and that imposed by the blood-purification protocol used in Chapter 2 to obtain activated neutrophil populations. Secondly, there are no published studies directly comparing cellular activation states occupied by the 'resting' and 'activated' neutrophil populations characterized in Chapter 2. The functional studies described in Chapter 3 address and resolve these issues by (i) analyzing the relative innate capacity of each neutrophil population to adhere to synthetic substrate in the absence of cellular stimulation with inflammatory ligand (e.g., fMLF) and (ii) examining the relative ability of each neutrophil population to activate and generate superoxide in response to fMLF-stimulation.

The subsequent studies described in Chapter 3 emphasize differences in membrane skeletal composition and structure that exist between resting and activated neutrophil populations. This comparison is facilitated by subcellular fractionation of N_2 cavitates of neutrophils using isopycnic sucrose density fractionation. The identification and characterization of membrane skeletons in density gradients is based on several criteria, including (i) the immunolocalization of primary structural components, actin, fodrin and ezrin relative to plasma membrane-specific and granule-specific marker activities; and (ii) the cosedimentation of primary structural components with select membrane anchoring proteins known to associate with and anchor each component to the plasma membrane bilayer. Compositional differences in membrane skeletal structure

detected in density gradients are further confirmed by compositional analysis of membrane skeletons isolated from detergent-extracted whole cells.

The final chapters, including Chapters 4 and 5, focus on novel antimicrobial and invasive properties conferred by dynamic adaptations in the plasma membrane structure and/or organization of human neutrophils and yeasts, respectively. In Chapter 4, a novel role of plasma membrane structure in regulating the localization and activity of the only human cathelicidin antimicrobial protein, hCap, is described. This work implicates the plasma membrane in compartmentalizing extracellular antimicrobial activity of degranulated proteins at the cellular-microbial interface, thereby minimizing associated host tissue damage occurring during the course of the neutrophil inflammatory response.

The capacity of hCap to bind plasma membranes was originally inferred from its relatively high immunogenicity in purified preparations of plasma membrane protein isolated from activated human neutrophils and used to elicit cytochrome b-specific monoclonal antibodies from normal BALB/c mice. Although the presence of hCap within such preparations was later found unrelated to the assembly and function of the NADPH oxidase complex, its presence on the cell surface suggested a novel property of the hCap holoprotein relevant to the spatial regulation of its antimicrobial function *in vivo*.

The work described in Chapter 4 presents a detailed characterization of hCap-plasma membrane localization properties and additionally examines the physical and biochemical nature of this association. Because accurate and conclusive identification of hCap as the immunogen of interest is foundational to this work, there is a recurrent emphasis placed on confirmation of hCap identity using multiple experimental methods.

This objective was accomplished in parallel with characterization studies using the same methods employed to examine hCap localization, including phage display analysis, isopycnic sucrose density sedimentation, fluorescence activated cell sorting (FACs), immunoprecipitation, and peptide mass mapping by matrix assisted laser desorption/ionization (MALDI) analysis. Collectively, the data presented in this study substantiate a significant cell-surface binding capacity of hCap that is likely mediated by an integral membrane protein and actively regulated by physiological properties unique to human neutrophils. The functional significance of these observations will be the subject of future investigations.

The concluding chapter, Chapter 5, examines the role of membrane cholesterol enrichment in modulating the *in vivo* survival and invasive properties of the fungal opportunist, *Candida glabrata*. This organism was originally isolated from specimen samples collected from a subpopulation of immune-compromised patients at University of Virginia School of Medicine, and later characterized and identified as the probable causative agent of the therapeutically resistant patient-liver and/or kidney complications that distinguished this subpopulation of patients. Significantly, this mutant organism cannot be identified from body fluids and/or tissue biopsy samples using the conventional media and culturing methods currently employed by clinical microbiologists. As a result, the organism remains undetected, and the primary cause of patient-pathology is mis-diagnosed and inadequately treated.

As cholesterol availability determines both the pathology and anatomical site of infection, these mutant strains are termed ‘bile-dependent’ and can only be cultured *in*

vitro, and thus detected in patient specimen samples, with a specialized media relatively enriched in cholesterol. Alterations in cholesterol metabolism necessarily affect various intracellular metabolic processes as well as the function and structure of cellular organelles. For example, congenital imbalances in lipid metabolism, commonly referred to as 'lipid storage disorders' often result in the accumulation of cellular lipids, including cholesterol, within vacuoles or lysosomes. Studies examining such disorders have found that lipid accumulation is generally toxic in eukaryotes. Therefore, the markedly enhanced requirement of bile-dependent strains of *Candida glabrata* for saturating concentrations of cholesterol is highly unusual and noteworthy. In addition, the vital role of cellular cholesterol homeostasis in the integrity, fluidity, organization, and structure of plasma membranes is well established and specifically relevant to the scope of material presented in this dissertation. Indeed, plasma membrane cholesterol facilitates and critically regulates both stimulus- and stress-induced signaling responses implicated in the pathogenesis of medically important fungi. Significant alterations in cellular cholesterol levels observed with bile-dependent *Candida glabrata* imply a unique molecular basis for the pathogenesis of this group of organisms *in vivo*. Hence, the work presented in Chapter 5 elucidates a novel etiological basis for disease in an otherwise well-characterized and intensely studied patient subpopulation. The work in this chapter, thus, emphasizes the relative importance of plasma membrane biochemistry, structure and organization, as well as the apparent genetic capacity of some opportunists, to the adaptation of organisms to specialized *in vivo* microenvironments that are otherwise hostile and generally uninhabitable by most other opportunistic microorganisms.

Background

The Plasma Membrane

The plasma membrane consists of a fluid core-component, the lipid bilayer that is penetrated by amphipathic integral membrane proteins. It is flanked intracellularly by the membrane skeleton, a complex molecular array of diverse protein-protein associations, and extracellularly by a carbohydrate matrix, or glycocalyx, derived from the sugar component of membrane glycoproteins and glycolipids (Mouritsen and Bloom, 1993) (Figure 1). Each layer of membrane structure contributes uniquely to the frictional drag exerted on passively diffusing transmembrane proteins, largely in terms of viscosity and steric effects, and can lead to the asymmetric distribution of surface receptor (Tsuji and Ohnishi, 1986;Sheetz, 1993). These properties, which are intrinsic to all biological membranes, have been previously discussed in terms of the Viscosity (Koppel et al., 1981), Percolation (Saxton, 1990a), and Transient-Binding models (Saxton, 1987;Saxton, 1990b;Saxton, 1990c) (Figure 2). The Viscosity Model describes the density effects of phospholipid and protein packing on the mobility of membrane proteins; the Percolation Model illustrates the barrier effect posed by the submembranous skeleton; and the Transient-Binding Model depicts the impact of nonspecific electrostatic interactions on protein diffusion. As implied in the latter Percolation and Transient-Binding models, the degree to which protein mobility is constrained or altogether prevented depends in part on the physical/biochemical properties of the protein under consideration, including its size and structure (Tsuji and Ohnishi, 1986;Sheetz, 1993). While protein's size may either increase or decrease the likelihood of collision with other membranous and/or

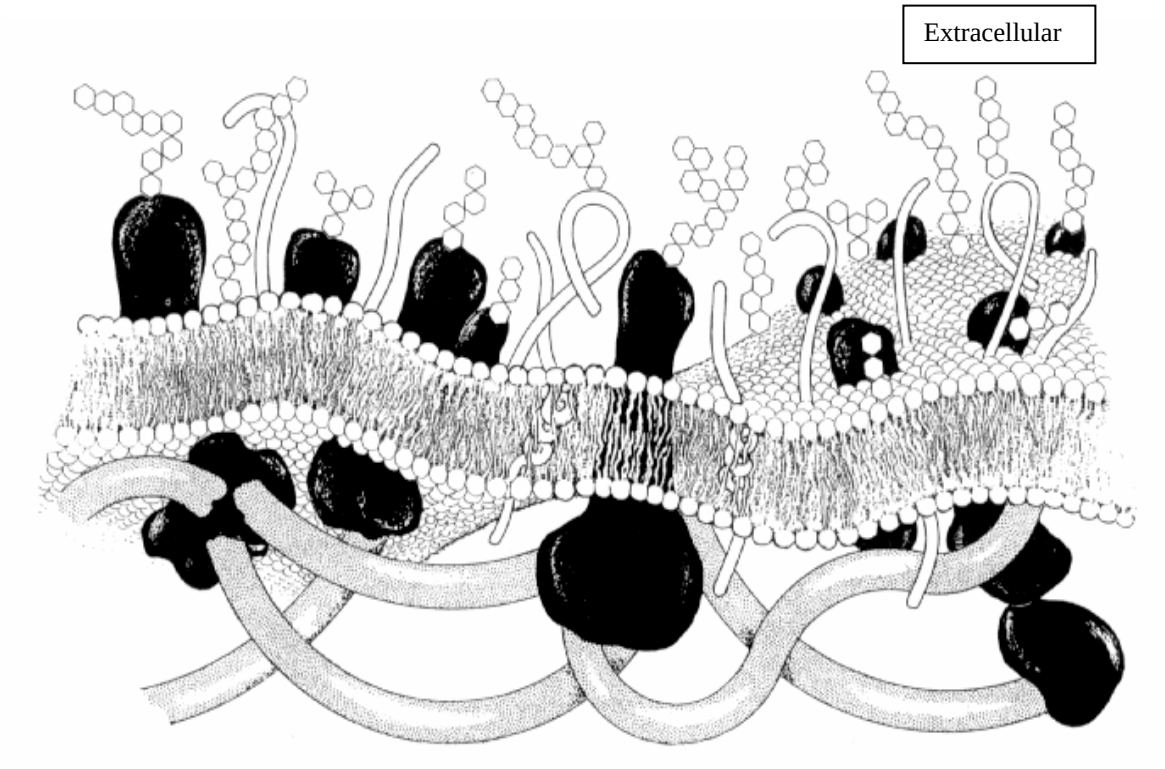


Figure 1. Structure of the eukaryotic cell plasma membrane. The fluid bilayer, densely packed with protein, is coated with an external sugar-matrix (hexagonal chains) and internally associated with an extensively complex, highly organized molecular framework (cable-like structures). The latter, generally referred to as the cytoskeleton, has long been recognized as a structural complex that fulfills necessary mechanical roles, such as the maintenance of cell shape; however, recent evidence indicates that this traditional view of cytoskeletal function is incomplete. In virtually all eukaryotes, the assembly of submembranous signaling complexes and/or the transfer of cell-signals has been inextricably linked to F-actin polymerization. One explanation for this aspect of cytoskeletal function is the dynamic assembly of physical domains, or “cytoskeletal fences,” which may localize activated membrane proteins into specialized subcellular pockets containing the cytosolic mediators necessary for signal transduction. This figure is from reference (Mouritsen and Bloom, 1993).

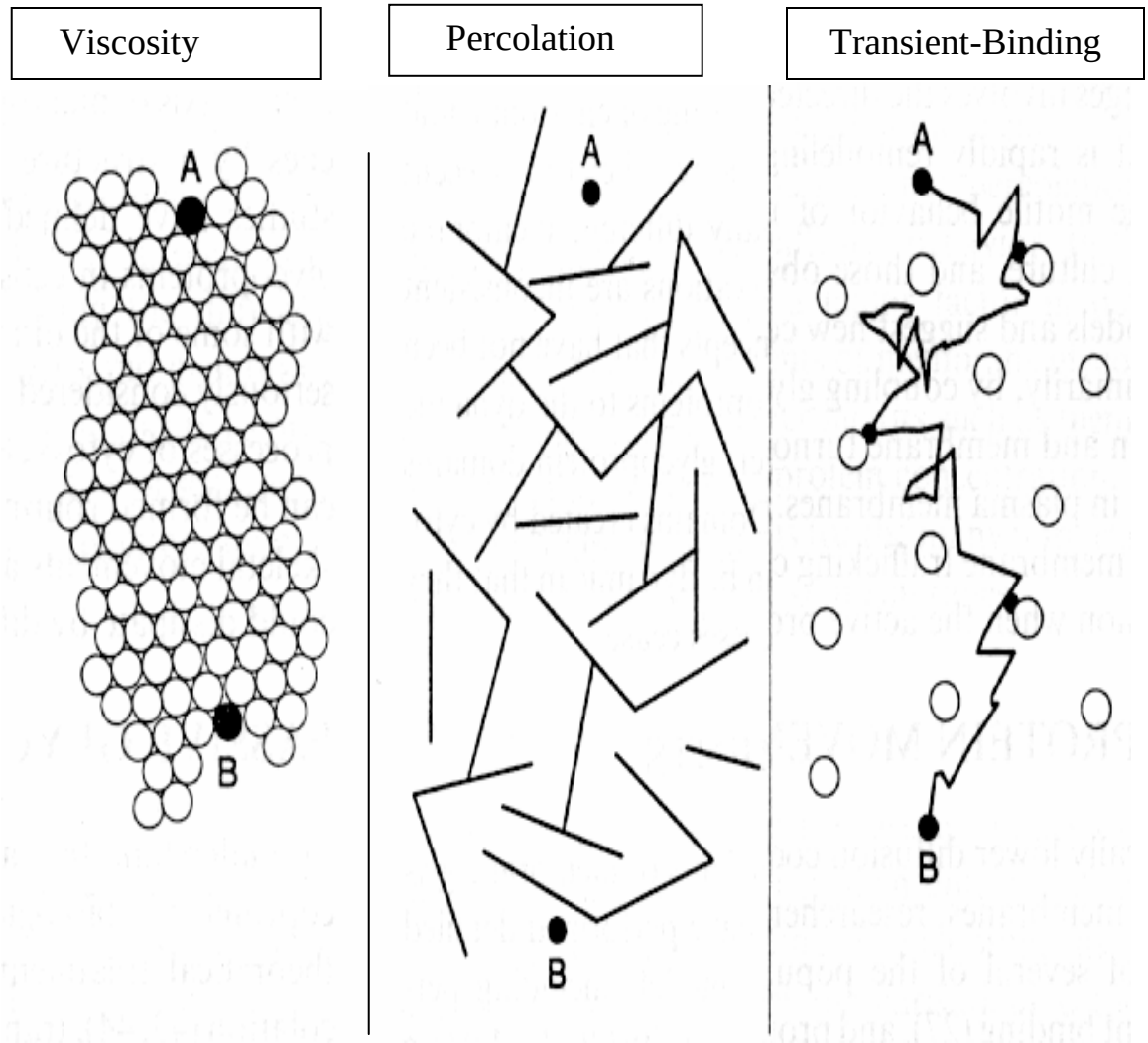


Figure 2. Regulation of membrane protein diffusion in plasma membranes. All biological membranes possess properties that inhibit the passive diffusion of membrane proteins. Depicted in this figure are three general models, which collectively explain these hindering forces. (left) The Viscosity Model demonstrates the effects of phospholipid-density and protein packing on the mobility of membrane proteins. (middle) The Percolation Model illustrates how the barrier effect of the submembranous skeleton may trap or otherwise impede the lateral motion of untethered proteins. (right) The Transient-Binding Model depicts how the lateral progress of charged or zwitterionic proteins can be impeded through nonspecific electrostatic interactions with other, bystander, proteins and/or charged cytoskeletal elements. Both the Percolation and Transient Binding models suggest that the physical/biochemical properties of membrane proteins influence their diffusion capability. In all cases, points A and B are the locations of a given protein before and after diffusion, respectively. This figure is from reference (Sheetz, 1993).

submembranous components, its structural features may confer the capacity to preferentially interact with certain classes of membrane phospholipids and/or select submembranous cytoskeletal elements, additionally affecting protein-function (Olorundare et al., 1993). Of the dynamic and diverse forces inherent in membrane structure that govern the mobility and/or activity of membrane proteins, protein-lipid and lipid-lipid interactions are especially relevant to the scope of this study.

Membrane Phospholipids. Traditional models of biological membranes, such as the Fluid Mosaic Model, have emphasized the structural role of phospholipid molecules (Singer and Nicolson, 1972). Affirming this point of view, phospholipid diffusion in pure lipid bilayers, which is primarily lateral (Kornberg and McConnell, 1971;Devaux and McConnell, 1972), and their incessant re-distribution contribute to the random arrangement of phospholipid classes within the plane of each leaflet. Such synthetic models suggest that interactions between neighboring plasma membrane phospholipids, though inherently nonspecific, are cooperative and function primarily to provide biological membranes with characteristic physical properties (Devaux, 1991). In this regard, however, typical bilayer paradigms contrast dramatically with biological membranes. Phospholipid trafficking in eukaryotic plasma membranes is remarkably dynamic, driven by constitutive, energy-dependent processes (Seigneuret and Devaux, 1984;Martin and Pagano, 1987). This energetically-driven remodeling of membrane phospholipids culminates in a carefully maintained lateral and/or transmembrane order that is acutely sensitive to perturbation (Schroit et al., 1985;McEvoy et al., 1986). In recent years, an increasing emphasis of research in the field of molecular membrane

biology has been the characterization of functional intermolecular associations of plasma membrane phospholipids. It is presently clear that the phospholipid composition of biological membranes fosters key lipid-lipid and/or lipid-protein interactions central to the physiological function of eukaryotes. Eukaryotic cells are generally characterized as having plasma membranes of defined composition. Four main classes of phospholipids, including phosphatidylserine (PS) > phosphatidylethanolamine (PE) > phosphatidylcholine (PC) > and sphingolipid (SL), are constitutive in erythrocyte plasma membranes, which approximate the membrane composition of most other eukaryotic cell-types (Schwartz et al., 1985;Devaux, 1991). The lipid topology of eukaryotic plasma membranes is largely defined by the activity of the membrane-associated enzyme, aminophospholipid translocase, which actively displaces aminophospholipids (PS and PE) to the inner leaflet (Comfurius et al., 1990). The functional significance underlying the transmembrane segregation of lipid classes is far-reaching, as intermolecular associations occurring between membrane phospholipids and/or cellular proteins affect processes central to maintenance of cell shape and viability, membrane trafficking, and the efficiency of signal transfer.

Cell Shape and Viability. The precision with which membrane phospholipids are distributed in eukaryotic plasma membranes implies that specifically defined asymmetrical environments are of general importance to membrane structure and cellular function (Devaux, 1991;Simons and Ikonen, 1997). The addition of exogenous lipids (Ferrell et al., 1985) or certain drugs (Truong et al., 1986;Moreau et al., 1997) to intact erythrocyte membranes affect transient changes in phospholipid distribution and

dramatically alter cell morphology, correlating cell shape with the phospholipid composition of the bilayer. Further, it has been suggested that the pleomorphic changes characteristic of aging red cells (Williamson and Schlegel, 1994) and the abnormalities in erythrocyte structure observed during certain disease states (Wilson et al., 1993) are due to the progressive loss of phospholipid asymmetry and can be linked to either the decreased efficiency or the complete inactivity of aminophospholipid translocase (Devaux, 1991;Ding et al., 2000;Quinn, 2002;Daleke, 2003). Such modifications in cell-shape are physiologically significant because they may destine cells for premature destruction (Zarkowsky et al., 1975). However, in addition to the aberrant morphological consequences that accompany changes in bilayer composition, functions vital to the maintenance of vascular physiology are also facilitated by slight changes in membrane order. For example, subsequent to vascular injury, PS redistribution to the outer leaflet of platelet plasma membranes potentiates fibrin deposition by accelerating thrombin formation at the lipid-protein interface of the so-called phospholipid complex (Bevers et al., 1983;Hemker et al., 1983;Zwaal and Bevers, 1983). Topological distortions caused by local disturbances in lipid order additionally serve as stimuli for constitutive endocytic processes and other membrane trafficking events (Holopainen et al., 2000). In many cases, these processes are driven by transient, local alterations in the phospholipid composition of plasma membranes.

As indicated above, the nonrandom arrangement of plasma membrane phospholipids results from the actions of membrane-bound enzymes, which catalyze the transfer of certain phospholipid classes from the outer leaflet to the inner leaflet (Tilley et

al., 1986). Accordingly, the characteristic transmembrane asymmetry typical of mammalian cell plasma membranes is constitutively maintained in an energy-dependant fashion (Williamson et al., 1987). However, recent research suggests that plasma membrane composition is less dynamic and more ordered than previously suspected (Friedrichson and Kurzchalia, 1998;Simons et al., 1999;Parasassi et al., 1999). One important consideration concerns the stability of local alterations in phospholipid composition. Such alterations are typically short-lived because they promote membrane stress and subsequent endocytosis (Dai et al., 1997). The aminophospholipids, PS and PE, are primarily involved in this aspect of membrane traffic due to their characteristic structure. In particular, the small polar head group and widely spaced acyl side chains of these phospholipid classes, when clustered, promote the inward, or negative, curvature of membranes (Zimmerberg et al., 1993;Jahn and Sudhof, 1999). The resultant bending of membranes, which requires energy, stimulates spontaneous endocytosis (Devaux, 1991;Jahn et al., 2003). Accordingly, the apparent transient nature of local concentrations of phospholipid classes is evidenced by both energetic and physiological constraints. However, a contrasting view of plasma membrane bilayers has recently emerged based on evidence indicating that certain phospholipids, the sphingosine-based lipids in particular, stably interact within the plane of outer leaflets (Brown and London, 1997;Iwabuchi et al., 2000;Handa et al., 2000;Yu et al., 2002;Hakomori and Handa, 2003;Tadano-Aritomi et al., 2003;Hakomori, 2003). This lateral clustering of phospholipids, which is contingent on the physical state of membranes (Hollan, 1996;London and Brown, 2000), suggests that leaflets consist of a heterogeneous

patchwork of free-floating, laterally diffusing compositional domains (Edidin and Stroynowski, 1991;Sako and Kusumi, 1994;Edidin et al., 1994;Feder et al., 1996;Anderson, 1998;Waugh et al., 1999;Wisniewska et al., 2003;Ritchie et al., 2003;Helms and Zurzolo, 2004).

Membrane Rafts: An Introduction. Evidence obtained from the biochemical analysis of purified plasma membranes has provided potential new insights into the intermolecular interactions of phospholipids in vivo and forms the basis of a recently proposed "ordered phase" (l_0) model known as the Raft Hypothesis (Brown and London, 1997). This theory suggests that the physical state of plasma membranes in vivo favors the aggregation of certain phospholipid classes within the plain of outer leaflets (Brown and London, 1997;Brown and London, 2000). As mentioned earlier, plasma membranes consist of hydrophobic and hydrophilic components (Mouritsen and Bloom, 1993). The hydrophobic component fluctuates between liquid (disordered), and solid, gel-like (well-ordered) states (Lee, 1977). In the gel-like state, membrane phospholipids are tightly packed, with acyl chains extended, whereas in the fluid state lipid molecules are kinked and loosely packed (London and Brown, 2000). The balance of order in membranes that exists between these two extremes is ostensibly complex and exceedingly difficult to predict. Transient, multifaceted interactions of biological membranes with numerous cytoplasmic and extracellular factors contribute to this complexity in vivo and can potentially alter the physical state of plasma membranes (Spector and Yorek, 1985). A variety of factors are known to influence the physical state of plasma membranes in vivo, including temperature (Brown and London, 2000) and cholesterol enrichment (Silvius et

al., 1996). The moderately high temperatures typical of in vivo conditions, combined with the high cholesterol content of mammalian cell plasma membranes (London and Brown, 2000), would appear to favor disordered rather than ordered states. Indeed, plasma membranes under in vivo conditions have been traditionally regarded as inherently disordered (Singer and Nicolson, 1972). However, there is evidence suggesting that plasma membranes adapt a semi-ordered state under *in vivo* conditions (Brown and London, 1997). In support of this view, liposome studies examining various lipid-cholesterol mixtures have found that cholesterol promotes phase separation (Silvius et al., 1996;Ahmed et al., 1997). In phase separation, leaflets assume a partial, semi-ordered state (l_0) that is intermediate between gel-like and liquid phases (Brown and London, 1997). Moreover, phase separation in semi-ordered membranes is characterized by the lateral partitioning of certain phospholipid classes into discreet, stable, freely diffusing aggregates (Xu and London, 2000). These lipid aggregates are small, rapidly mobile entities that may be randomly dispersed throughout leaflets, in which the major lipid fraction is predominantly disordered, consisting of freely diffusing individual phospholipids (Varma and Mayor, 1998). Stable intermolecular associations between phospholipids are cholesterol-dependent. Thus, the cholesterol component of mammalian cell plasma membranes may contribute to the intrinsic heterogeneity of membranes by engendering phase separation (Brown and London, 1997). The molecular nature of cholesterol-dependent, intermolecular phospholipid associations is thought to occur through electrostatic (head-group) and hydrophobic (acyl chains, cholesterol) associations (Simons and Ikonen, 1997), as depicted in Figure 6. Sphingolipids are the

major class of phospholipid that contribute to the structure of membrane domains because their long, saturated fatty acid chains pack tightly together in the presence of cholesterol and thereby contribute to the stability of microdomains (Brown and London, 2000). In the absence of cholesterol, the relative bulk of the head groups do not permit lateral associations between the acyl chains of adjacent phospholipids (Simons and Ikonen, 1997). In this situation, phospholipids interact exclusively through their charged headgroups and therefore fail to establish strong, cohesive associations (London and Brown, 2000) (Figure 6). Cholesterol, however, inserts between neighboring lipid molecules, bridging the gap between phospholipids and thereby stabilizing lipid-lipid associations (Simons and Ikonen, 1997). Accordingly, cholesterol has been postulated as "molecular glue," without which microdomains would fail to form (Fielding and Fielding, 2000). Though strong evidence for the existence of microdomains in vivo is lacking (Chen et al., 1997;Kenworthy and Edidin, 1998) (Jacobson and Dietrich, 1999), persuasive, though indirect, evidence for their existence has been obtained.

Evidence supporting the existence of compositional microdomains has been primarily based on their isolation following the in vitro extraction of detergent-solubilized plasma membranes. The biochemical isolation of such domains is made possible due to their distinct physical properties relative to the surrounding membrane. Specifically, microdomains are traditionally defined by their (a) insolubility in Triton X-100 (Sargiacomo et al., 1993;Schroeder et al., 1998;Hooper, 1999;Barenholz, 2004); (b) low density following isopycnic centrifugation (Schnitzer et al., 1995;Smart et al.,

1995;Song et al., 1996); and (c) selective enrichment of sphingosine-based lipids (Kojima and Hakomori, 1991;Kojima et al., 1992;Iwabuchi et al., 1998;Hakomori et al., 1998a;Hakomori et al., 1998b;Hakomori, 2004). The latter criterion concerns the issue of phospholipid packing. As mentioned above, saturated acyl chains pack tightly in the presence of cholesterol, whereas unsaturated chains do not. The hydrophobic aspect of intermolecular phospholipid association is proposed as the most critical to the formation of microdomains (Simons and Ikonen, 1997), as evidenced by types of lipids that have been isolated to date from these structures. The concept of raft-detergent insolubility has been recently expanded by studies examining membrane solubility in a host of different detergents in an attempt to segregate compositionally distinct raft assemblies from the more nonspecific, or 'bulk,' isolation methods employing Triton X-100 (Shogomori and Brown, 2003;Chamberlain, 2004;Gombos et al., 2004;Locke et al., 2005). Lipid molecules that frequently coisolate with compositional domains include gangliosides (Harder et al., 1998;Villar et al., 1999), sphingomyelin (Dobrowsky, 2000), ceramide (Smaby et al., 1996;Anderson, 1998), diacylglycerol (Magee et al., 2002;Schmitz et al., 2003), and cholesterol (Simionescu et al., 1983;Dietrich et al., 2001;Nutikka and Lingwood, 2004;Megha and London, 2004). Though composed of only a few phospholipid classes, compositional domains vary remarkably in size and morphology [(Anderson, 1998;Subczynski and Kusumi, 2003) (Figures 3, 4, and 5)]. Regarding the latter, domains may be flat, vesicular, or tubular (Parton and Richards, 2003;Stan, 2005;Li et al., 2005). Partially invaginated domains may be flask-shaped or rounded, both of which may be open at the cell surface or closed off.

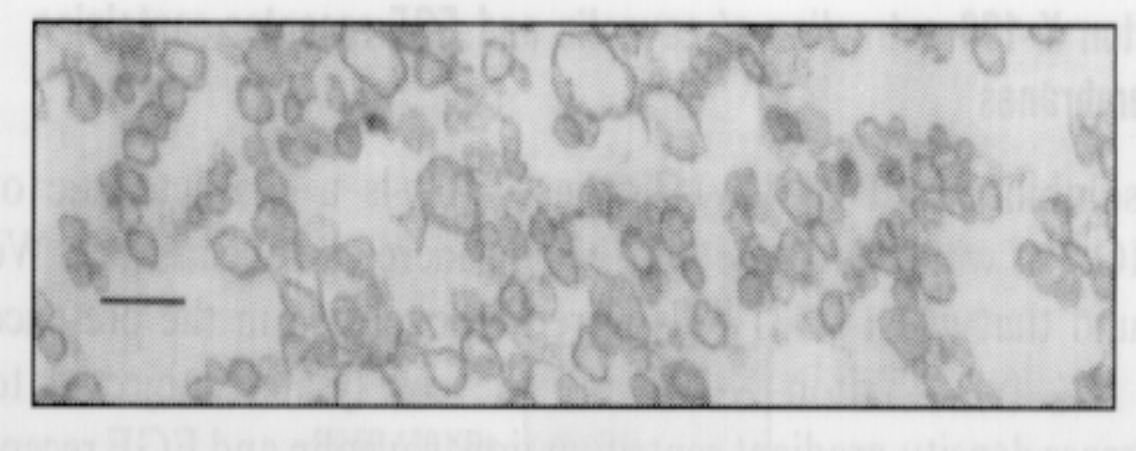


Figure 3. Detergent-insoluble lipid vesicles present in light density regions of cell lysates fractionated by density gradient sedimentation. These light density lipid vesicles represent membrane rafts. The consolidated (tightly bound) lipid structure of rafts confers detergent insolubility, which in turn underlies their characteristic localization properties within light density gradient regions. Many studies indicate that membrane rafts assembly occurs preferentially in activated cells. Such conditional raft assembly is consistent with the proposed primary role of rafts in transducing cellular signals. Therefore, in order to obtain the light density membrane fraction shown in the above micrograph, cells were first activated by exposure to a specific receptor-agonist. Cells were then disrupted by one of several mechanical procedures that vesiculate plasma membranes but leave internal organelles intact, followed by the removal of free nuclei from cellular lysates by low speed sedimentation. This nuclei-free cellular lysate is sometimes referred to as the post-nuclear fraction. This post nuclear fraction is transferred to the bottom of a sedimentation tube and overlaid with a linear or step gradient solution. Detergent-insoluble membrane fractions will float up through the gradient material until it reaches equilibrium within light density regions of the gradient. The above is an electron micrograph, taken by Waugh et al., depicts the low density fraction obtained by isopycnic centrifugation of detergent insoluble membrane fractions. The spherical structures represent individual domains, which vary remarkably in size. This figure is from reference (9).

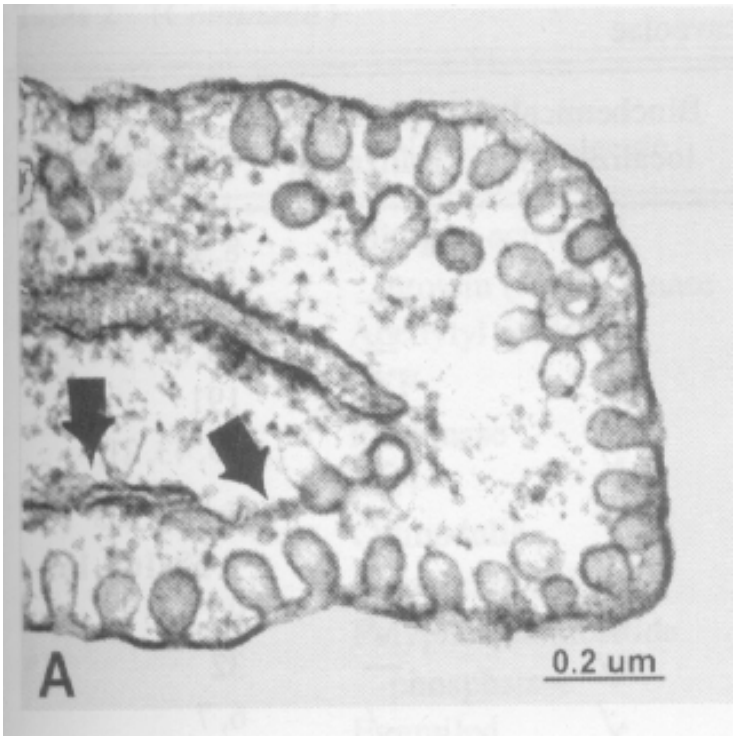


Figure 4. Caveolae morphology in the mammalian cell plasma membrane. Caveolae are microdomains enriched in saturated sphingosine-based phospholipids, doubly transacylated cytosolic signaling kinases and GTPases, and caveolin. Caveolin is arguably the most crucial constituent of membrane caveolae. A uniquely hairpin-shaped molecule, caveolin is a transmembrane protein that, upon insertion in plasma membranes, promotes local distortion of bilayer morphology. Prior to their delivery and insertion into plasma membranes, caveolin associates with newly synthesized cholesterol from membranes of the endoplasmic reticulum (ER; arrows) in a high affinity bimolecular complex. In this way, caveolin not only serves as a primary chaperone activity involved in the intracellular cycling of cholesterol from their site of synthesis in smooth ER membranes to the cell surface but also is thought to be a key component of the efflux mechanisms responsible for the delivery of cellular cholesterol to high-density lipoprotein and other plasma lipoproteins. Caveolin expression is primarily confined to non-hematopoietic stromal, vascular and tissue cells, including fibroblasts, endothelial and tissue cells. Following the disposition of caveolin within the plasma membrane, the intermolecular clustering of bimolecular caveolin-cholesterol complexes bends the membrane bilayer inward in vesicular-shaped invaginations that morphologically define membrane caveolae. A thin section of plasma membrane decorated with numerous flask-shaped caveolae is represented in the above micrograph from experiments performed using electron microscopy. This figure is from reference (Anderson, 1998).

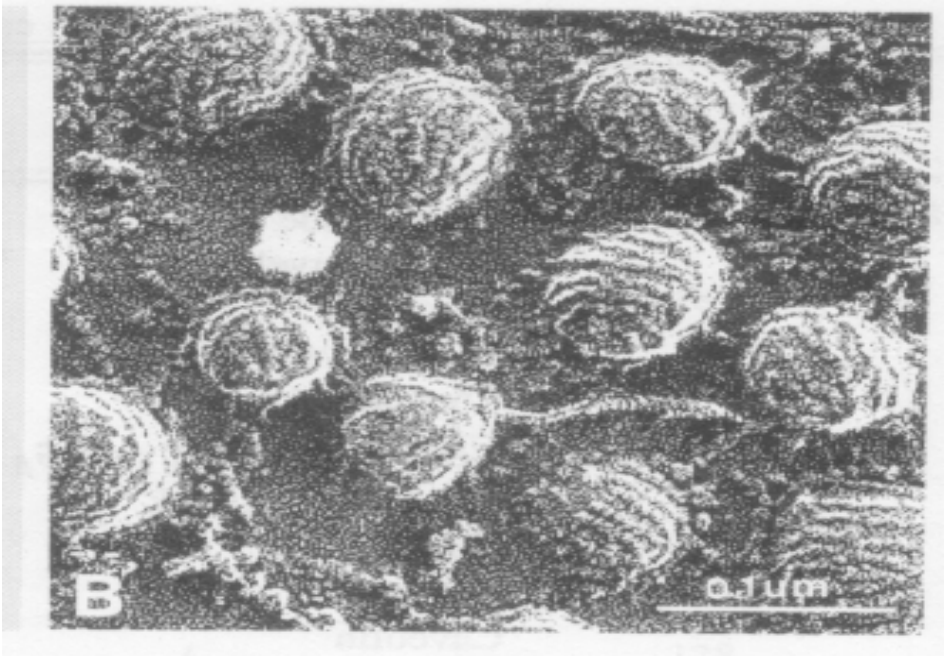


Figure 5. Morphology of caveolae-enriched, mammalian cell plasma membranes. The above is a rapid-freeze deep-etch image detailing the internal, cytoplasmic surface of the plasma membrane. The invaginations depicted are ~ 50 nm in diameter and appear evenly striated, due to the specialized topology is characteristic of caveolin organization within the caveolae of certain cells. Although caveolin expression is generally required for caveolae assembly in mammalian cell surface membranes, striated caveolae appear to be cell-specific variations observed mostly in stromal cells. Interestingly, such observed variations in the shape and/or organization of caveolae between different classes of mammalian cells are not associated alterations in their primary functional properties, which include membrane trafficking, cholesterol transport, and signal transduction. The morphological and functional properties of caveolae are derived from their protein composition. As such, caveolae essentially comprise specialized ‘membrane pockets’ also referred to as membrane ‘microdomains.’ The microdomains depicted in Figures 3-5 are, by definition, plasma membrane regions of a molecular composition and/or function that differs from the surrounding membrane. This figure is from reference (Anderson, 1998).

Protein association greatly influences the shape of a given domain. For example, phospholipid aggregates that interact with caveolin, a self-associating integral membrane protein, take on a crater-like morphology (Schlegel et al., 1998) (Figure 5). Caveolin association results in the formation of characteristic striations (Peters et al., 1985) that are visible in the micrograph presented in Figure 5. In addition to their morphological diversity, recent data indicate that lipid microdomains are equally diverse in composition. For example, the results obtained from certain studies suggest that certain lipid microdomains can exist in the absence of sphingosine-based lipids (Mendez et al., 2000) (Ostermeyer et al., 1999). Moreover, as implied above, microdomains may or may not associate with caveolin (Arvanitis et al., 2005;Gaus et al., 2005). As indicated in Figure 5, caveolin association induces a characteristic crater-like morphology, whereas caveolin poor microdomains lack curvature. Regardless of their morphological and compositional diversity, however, lipid microdomains, once formed, comprise a distinct membrane system that fulfills specific roles essential for normal cellular function (Anderson, 1998).

Raft Formation and Cell Polarization. Phospholipid asymmetry drives the aggregation of certain phospholipid classes in cell membranes, creating phospholipid (or membrane) rafts, which are important in membrane trafficking events and cell signaling processes (Simons and Ikonen, 1997). The plasma membrane, constantly recycled through constituative endocytic and exocytic vesicular traffic, is supplied with phospholipid and protein by the trans-Golgi network (Bishop and Bell, 1988). In epithelial cell lines, phospholipid rafts first assemble in the outer leaflet of trans-Golgi

membranes, where they may serve as a signal that destines nascent proteins for deposition into apical domains of adherent cell monolayers (Ikonen and Simons, 1998;Lafont et al., 1999). It has become evident in recent years that the phospholipid topology of Golgi membranes is uniquely capable of supporting the formation of rafts (Hansen et al., 2000). Membrane rafts are rich in glycosphingolipids, synthesized in the Golgi, and cholesterol, synthesized in the endoplasmic reticulum (ER) (Yusuf et al., 1983;Kaplan and Simoni, 1985). Cholesterol is deposited into Golgi membranes as a result of vesicular traffic from the ER (Bishop and Bell, 1988). The phospholipid composition and cholesterol content of Golgi bilayers is strictly maintained in order to support the dynamic assembly of rafts, which initially occurs in the trans Golgi network (TGN) (Simons and Ikonen, 1997). Vesicular traffic from the TGN to plasma membranes occurs on a selective basis in polarized cells. Whether nascent proteins are deposited into basal or apical membranes depends on specific sorting signals. For example, glycosylphosphatidylinositol (GPI)-anchored proteins (Lisanti et al., 1988) and certain transmembrane proteins (Kundu et al., 1996) are targeted to apical membranes by their ability to associate with membrane rafts. In the latter case, proteins may possess intrinsic signals, such as specific amino acid sequences, in their transmembrane domains that allow them to partition into rafts (Kundu et al., 1996); also, the N-glycans present on glycosylated proteins allow partitioning into rafts (Scheiffele et al., 1995). On the other hand, the basolateral targeting of proteins requires the presence of a specific amino acid signal in their cytoplasmic domain, such as a tyrosine or a dileucine motif (Matter and Mellman, 1994). Like apical proteins, basolaterally targeted proteins can be glycosylated;

in such a case, it is thought that the basolateral signal housed in the cytoplasmic domain is stronger than the apical-targeting signal generated by the N-glycans (Matter and Mellman, 1994;Scheiffele et al., 1995). Such selective targeting processes results in apical and basal surfaces that are unique in terms of protein content and phospholipid composition (Rodriguez-Boulan and Nelson, 1989). Membrane proteins and lipids remain segregated due to the presence of tight junctions between adjacent cells (Rodriguez-Boulan and Nelson, 1989;Cereijido et al., 1998). These junctions prevent the lateral diffusion of proteins and lipids from one domain into another. It follows, therefore, that the plasma membranes of polarized cells are organized laterally, due to the barrier effect of tight junctions, in addition to the normal transmembrane organization of lipids (Simons and Ikonen, 1997). Regarding the lateral organization of polarized cells, simple glycosphingolipids, such as glucosylceramide, and sphingomyelin are concentrated on the outer leaflet of apical membranes (Simons and van Meer, 1988), and the glycerophospholipid class, PC, is concentrated in the outer leaflet of basolateral membranes (van Meer, 1989).

Membrane rafts are resident in the outer leaflet of apical plasma membranes and function as free-floating platforms capable of selectively interacting with cytosolic signaling proteins (Fessler et al., 2004). Only the outer leaflet of raft structure has been compositionally defined. The existence of an inner leaflet component of raft structure is controversial primarily because the hydrocarbon chains of the aminophospholipid classes present in the inner leaflet (PS and PE) are mostly polyunsaturated. Nonetheless, other, minor phospholipid classes present in the inner leaflet, such as phosphoinositides, have a

hydrocarbon structure that would support a stable transbilayer association with raft lipids. Results from at least one recent study (Pyenta et al., 2001) indicate that membrane rafts in nonactivated plasma membranes lack an organized inner leaflet structure but, upon cellular activation, rapidly acquire an inner leaflet phospholipid composition that is capable of supporting the raft-localization of various cytosolic enzymes.

Although the lipid component of raft structure is essential, rafts may vary according to protein composition (Aman and Ravichandran, 2000;Tansey et al., 2000;Funatsu et al., 2000;Nusrat et al., 2000;Lockwich et al., 2000).The various associated proteins from which rafts are composed determine their functional significance (Brown and London, 2000). Raft-protein associations may be inducible (Lou et al., 2000) or constitutive (Varma and Mayor, 1998;Lipardi et al., 2000). Proteins capable of associating with rafts include, GPI-anchored proteins (Kasahara and Sanai, 1999), effector proteins such as acylated tyrosine kinases of the Src family (Holowka et al., 2000), and transmembrane proteins [(Skibbens et al., 1989) (Figure 6)]. Raft-associated GPI-anchored proteins are constitutively associated with rafts and may facilitate the transmission of cell signals in several ways. Though extracellular, they can be cleaved enzymatically into active second messenger phospho-oligosaccharides, which can then be transported into the cytosol (Parpal et al., 1995). In addition, antibodies specific for GPI-anchored proteins co-precipitate with Src family kinases, indicating the participation of membrane rafts in kinase-activated pathways (Miotti et al., 2000). Evidence also indicates that certain transmembrane proteins preferentially localize to rafts. One recent example is the FcεR1 receptor present in the plasma membrane of mast cells (Figure 7).

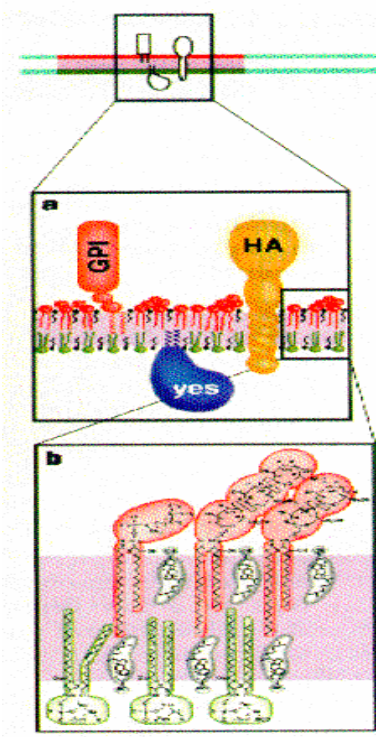


Figure 6. The molecular composition, structure and localization of membrane rafts. Membrane rafts (red) exist on the apical surface of adherent epithelial cells and represent specialized domains of the outer leaflet that are structurally distinct from the remaining bilayer. (A) Shown are proteins that normally associate with rafts. Glycosylphosphatidylinositol (GPI)-anchored proteins are extracellular and associate with the phospholipid component of rafts. Doubly acylated tyrosine kinases such as YES, a Src family kinase, insert into the cytoplasmic leaflet directly beneath raft structures. Transmembrane proteins such as influenza virus haemagglutinin (HA) interact with rafts through their transmembrane aspect. (B) Rafts structure derives from the lateral association of glycosphingolipids (red) which interact, electrostatically, by way of their bulky carbohydrate head groups. The gaps between associating phospholipids are filled by cholesterol (gray), which is key to the stabilization of raft structure. In addition, the marked lateral and transmembrane segregation of phospholipids can be appreciated by noting the lateral clusters in conjunction with the color contrast of inner and outer leaflets: glycerolipids (green), present in the inner leaflet, underlie glycosphingolipid (red)-cholesterol (gray) aggregates. (C) Caveolae form in plasma membranes as a result of the caveolin-cholesterol associations, which accounts for the presence of this protein in raft-rich fractions obtained from subcellular fractionation studies. Self-associating caveolin molecules form hairpin loops that bend the bilayer inward, forming crater-like invaginations inherent in the surface topology of most eukaryotic species; hence, this inward bending of the bilayer is unique in that it is essentially stable and does not trigger endocytic traffic. This figure is from reference (Simons and Ikonen, 1997).

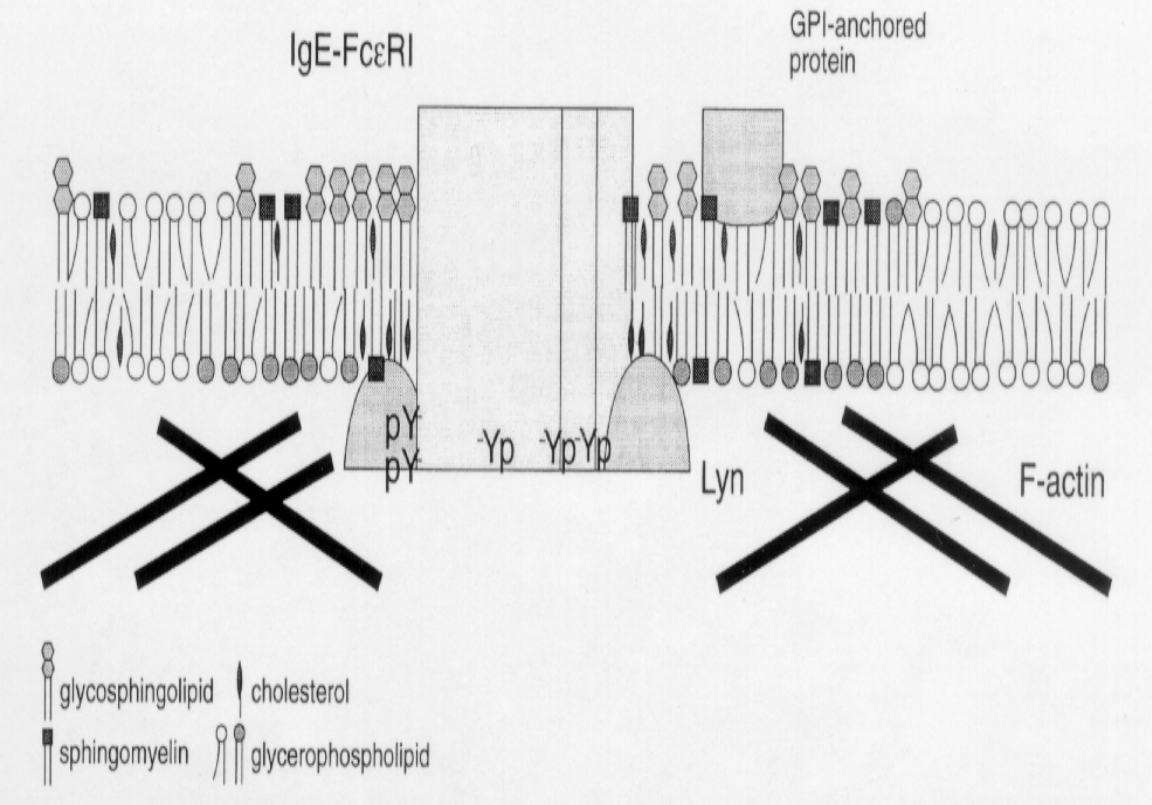


Figure 7. IgE-FcεR1 signaling in human leukocytes. Shown above is a recently proposed model describing IgE-FcεR1 signaling in the context of protein-lipid interactions. The concept of lipid-mediated signal transfer is premised on the formation of glycosphingolipid-cholesterol enriched complexes in the plane of outer apical leaflets. Referred to as “rafts,” these free-floating lipid aggregates function as platforms, to which acylated, cytosolic signaling molecules are selectively translocated. Accordingly, the above model describes a signaling process that is dependent on the segregation of activated surface receptor into glycolipid enriched domains of apical plasma membranes. In addition, cross-linked receptors do not aggregate when plasma membranes are depleted of cholesterol, further emphasizing the role that membrane structure plays in IgE-FcεR1 signaling. This illustration is from reference (Sheets et al., 1999a).

In this instance, the coalescence of raft structures may facilitate the aggregation of crosslinked surface receptors (Sheets et al., 1999b;Lafont and Simons, 2001;Kovarova et al., 2001) and couple activated receptors to downstream signaling kinases (Holowka and Baird, 2001) (Figs 6-7). In addition, certain studies suggest that certain heptahelical receptors, upon activation, associate with specialized raft-like compositional domains (Li et al., 1995). Given that phospholipid rafts constitute a major signal for the targeting of membrane proteins to the apical surface, signaling proteins resident in the apical surface could share similar affinity for raft structures. It is therefore plausible that apical proteins are generally housed in preformed microdomains. One primary point of interest concerns the existence of rafts in non-epithelial cell lines, CHO cells in particular.

Raft Formation in Non-Polarized Cells. Previous work has established that fibroblasts, a non-epithelial cell line, utilize trans Golgi network-derived membrane rafts to target proteins to the plasma membrane (Musch et al., 1996). CHO cells, though an embryonic cell line, are essentially fibroblast-like, both physiologically (Tsuji and Miyama, 1992) and morphologically (O'Neill and Hsie, 1978), suggesting that this cell-type may target nascent proteins to apical and basolateral membranes by similar signals. Indeed, evidence indicates this to be the case (Yoshimori et al., 1996).

Several recent studies have confirmed the presence of raft-like microdomains in the plasma membrane of several non-polarized cell-lines (Harder et al., 1998) (Varma and Mayor, 1998). Nonetheless, the process of cell polarization may, itself, modulate the types of membrane rafts formed, as well as their function and ubiquity in cellular membranes. In particular, the polarized morphology of epithelial cell monolayers

promotes the maintenance of a lateral asymmetry of phospholipid classes between apical and basolateral domains. This, in turn, may provide an environment that promotes the formation, maintenance, and function of membrane rafts. For example, rafts preferentially accumulate in the apical membranes of polarized cells (Verkade and Simons, 1997), which form tight, lateral junctions (Rodriguez-Boulan and Nelson, 1989), whereas in nonpolarized phenotypes rafts distribute over the entire cell surface (Varma and Mayor, 1998). Cell polarization may therefore sufficiently concentrate raft components in the apical membranes of epithelial cells so as to allow for their spontaneous or regulated coalescence and/or disassembly. However, in view of evidence which implies a requirement for the lateral clustering of raft structures in the plane of the outer leaflet prior to the initiation of raft-mediated signaling events (Baird et al., 1999;Janes et al., 1999), it is evident that nonpolarized phenotypes are intrinsically capable of regulating the spatial organization of membrane rafts on the cell the surface, despite the absence tight junctions.

Rafts are widely recognized as signaling platforms that preferentially cluster signaling proteins in a pathway specific manner (Shimada et al., 2005;Nika et al., 2006;Tanimura et al., 2006;Omidvar et al., 2006). Evidence that rafts function similarly in both nonpolarized (Menon et al., 1984;Sheets et al., 1999c;Holowka et al., 2000), and polarized (Kramer et al., 1999;Tansey et al., 2000) cells is apparent from independent investigations, suggesting that the dynamics of raft assembly and disassembly in plasma membranes may not be contingent on cell polarization. Such findings raise the question

of whether significant differences in the concentration of plasma membrane rafts exist on the apical surfaces of polarized and nonpolarized cells. Indeed, the excessive accumulation of membrane rafts within the apical membranes of epithelial cells may be dampened by their regulated endocytosis from the cell surface (Hansen et al., 1999). Accordingly, raft distribution, even if highly dispersed as is likely in the in the case of nonpolarized phenotypes (Kenworthy and Edidin, 1998), may not detract from their participation in signal transfer events. Moreover, recent findings indicate that raft structures are actively mobilized into membranous regions where activated receptors are localized (Manes et al., 1999). This active transport process may involve the activation of raft-localized kinases (Lou et al., 2000), possibly in conjunction with phosphoinositide-initiated actin polymerization (Rozelle et al., 2000). Of relevance to this study is the possibility that such mechanisms mobilize and concentrate glycosphingolipid classes in the outer apical leaflets of adherent, nonpolarized cells in order to permit the dynamic assembly and disassembly of raft-like compositional domains. In this regard, it is interesting to note that distinct, pre-existing lateral domains of defined protein and lipid composition are present in neutrophil plasma membranes (Jesaitis et al., 1988). The sequestration of certain surface receptors into such domains provides a mechanistic basis for the regulation receptor activity (Jesaitis et al., 1988). Thus, in view of the evidence presented above and given the diverse range of proteins that contribute to raft structure (Schroeder et al., 1998;Wollscheid et al., 2004;Blonder et al., 2004), their ubiquity in apical membranes could provide a general mechanism of receptor regulation as well as a means of signal transduction by which polarized and nonpolarized cells alike avoid crosstalk and preserve signal specificity.

Polyphosphatidylinositides. One additional consideration regarding the organization of phospholipid classes in plasma membranes is the apparent intrinsic capacity of mammalian cells to concentrate certain rare classes of signaling lipids into discrete membranous regions (Caroni, 2001;Meiri, 2004;Huang et al., 2004;Golub and Pico, 2005). Phosphatidylinositols (PI) and their metabolites serve as substrates, cofactors, and docking targets for membrane-partitioning cytosolic effector molecules that function downstream of all major receptor subclasses, most notably receptor tyrosine kinases (Yart et al., 2000), receptor associated tyrosine kinases (Ueno et al., 1998), and the G-protein subunits, alpha and beta-gamma (Naga Prasad et al., 2000), which couple to seven- transmembrane G protein-coupled receptors. Thus, membrane phosphoinositides independently regulate a wide-range of receptor-mediated cellular activities. Indeed, the importance of this phospholipid class in the pathology of human disease is well established, as metabolic disorders in phosphoinositide metabolism promote tumorigenesis and/or the metastasis of established tumors (Balla, 2001;Balla, 2005).

In mammalian cells, the synthesis and turnover of PI is strictly controlled. It comprises from one to ten percent of the total phospholipids and, regarding the plasma membrane, is predominantly found in the inner leaflet (Devaux, 1991;Janmey et al., 1999). The PI-content of cell membranes appears to be universally governed by a steady-state mechanism under the control of various kinases and phosphatases (Toker, 2002). However, not withstanding the rapid lateral mobility of plasma membrane phospholipids

(Ladha et al., 1996;Dietrich et al., 1997;Harms et al., 1999;Gaede and Gawrisch, 2003), the rapid turnover of inositol lipids following cell activation suggests the existence ofpreformed pools of this lipid class in plasma membranes (Pike and Miller, 1998;Meyer et al., 2001;Bian et al., 2001;Cho et al., 2002;van Rheenen et al., 2005;Cho et al., 2005a;Cho et al., 2005b). One study has shown that cholesterol depletion from plasma membranes inhibits hormone-stimulated PI-turnover in fibroblasts (Pike and Miller, 1998). One likely interpretation of this occurrence is the delocalization of PIP_2 from rafts (Brown and London, 2000). In agreement with this interpretation, PI-directed phospholipases are found to localize to PI-enriched microdomains following cell activation (Villalba et al., 2002;Dienz et al., 2003;Altman et al., 2004). Accordingly, it has been found that polyphosphoinositides, PIP_2 in particular, preferentially accumulate in regions of the plasma membrane that are enriched in cholesterol and sphingolipids.

Caveolae are cholesterol-enriched, shallow, crater-like invaginations found in plasma membranes that vary in diameter from 50 to 100nm (Kurzchalia and Parton, 1999). While membrane rafts may or may not partition into caveoli (Fra et al., 1994;Dobrowsky, 2000), protein kinases and certain PI-metabolites are commonly found in these specialized structures (Pike and Miller, 1998). Moreover, PI-kinases and their phosphoinositide substrates co-isolate with the cytoskeleton in detergent-solubilized cells (Janmey et al., 1999), and release of phosphoinositides from this fraction followed by protease treatment (Vickers et al., 1987). In addition, PI kinases are also found capable of tightly interacting with scaffolding proteins (Vickers et al., 1987;Payrastre et al., 1992). Altogether, this evidence supports the notion that inositol lipid-enriched microdomains of

plasma membranes are additionally associated with submembranous regions in which PI kinases are physically linked to the cytoskeleton. Indeed, the formation of stable, nondiffusible pools of phosphoinositides appears to be required for their proper function in certain pathways (Gilmore and Burridge, 1996;Jost et al., 1998;Nomoto et al., 1998;Janmey et al., 1999;Weernink et al., 2000;Sundaram et al., 2004;Haucke, 2005;Xie et al., 2005) and further indicates that the appearance of PI kinases in detergent insoluble fractions results from physiologically based associations with the cytoskeleton.

Inositol lipids additionally function as effector molecules and are important regulators of cytosolic and membrane associated proteins that modulate actin polymerization (Mejean et al., 1995;Barkalow et al., 1996;Gilmore and Burridge, 1996;Janmey et al., 1999;Rozelle et al., 2000;Suchy and Nussbaum, 2002;Xian and Janmey, 2002;Chou et al., 2002;Corgan et al., 2004). The activity of some effector molecules targeted by phosphoinositides is contingent on the amount of available lipid. For example, PIP_2 modulates cytoskeletal rearrangement by directly interacting with a diverse array of actin-binding proteins, including those that inhibit and/or promote polymerization (Schmidt and Hall, 1998). One of the ways in which PIP_2 promotes the formation and stabilization of cytoskeletal actin polymers is by binding and sequestering profilin, a protein that inhibits polymerization by binding the rapid-growing end of actin monomers but also, in complex with PIP_2, nucleates actin monomers to form F-actin (Lassing and Lindberg, 1985;Lassing and Lindberg, 1988a). When PIP_2 is incorporated into liposomes at concentrations below five to ten percent (total phospholipid) and are then added to profilin-actin complexes, these complexes remain intact. Conversely, when

the amount of PIP_2 surpasses ten percent of total lipid, PIP_2-profilin complexes form rapidly and actin polymerization occurs(Lassing and Lindberg, 1985;Lassing and Lindberg, 1988b). A similar regulatory dependence on PIP_2 is observed with gelsolin, another protein capable of promoting the disassembly and assembly of actin polymers (Janmey and Stossel, 1989;Wang et al., 2002a;Srinivasan et al., 2003). It therefore appears that the presence of the appropriate phosphoinositide, randomly distributed in membranes, is not sufficient to promote the activation of effector molecules, some of which (profilin) may be required for key cellular processes, such as cell motility (Lassing and Lindberg, 1988a). Accordingly, evidence indicates the physiological importance of phosphoinositide packing in cellular membranes and, in particular, that phosphoinositide-directed recruitment of sigaling and effector proteins contributes to remodeling of the F-actin cytoskeleton as well as to F-actin-dependent cellular processes, such as chemotaxis and adhesion.

Some investigators have described two distinct populations of PIP_2 in erythrocyteplasma membranes on the basis of phospholipase activity, including phospholipase-protected PIP_2 pools and phospholipase-targeted PIP_2 pools (Gascard et al., 1993). In this study (Gascard et al., 1993), it was found that both phosphatidylinositol 4-phosphate (PIP) and PIP_2 coexist in each population. Interestingly, the two populations become indistinguishable following the removal of skeletal proteins from membranes, as the formerly protected population acquired protease sensitivity. It follows that certain phosphoinositides pools are subject to sequestration, which may occur through direct or indirect phospholipid-cortical protein associations. Such phospholipid-protein

interactions potentially underlie the localization of phosphoinositides present within the inner leaflet of the nuclear envelope into discreet, kinase-rich regions of the nuclearmatrix (Cocco et al., 1987;Divecha et al., 1991;Payrastre et al., 1992). Such studies indicate a clear subcellular organization of pre-formed cellular phosphoinositide pools. Additional studies further suggest that phosphoinoside metabolism is intrinsically coupled to changes in cellular activation state. For example, in human platelets, the amount of PIP_2 extracted by chloroform/methanol decreased as a result of thrombin-mediated activation and platelet aggregation, thus indicating the integrated nature of PIP_2-metabolism in the context of cellular function (Vickers et al., 1987).

Collectively, the above evidence favors a view in which inositol lipids areselectively concentrated into microdomains within plasma membranes which in turn associate with cortical regions that support the localization and attachment of PI kinases. As previously mentioned, it is well established that PIP_2 physically interacts with numerous actin-binding proteins of the cell cortex to affect cytoskeletal rearrangement (Schmidt and Hall, 1998). The mechanisms by which PI and cortical protein interactions modulate the cytoskeleton are thought to involve dual-functioning proteins, such as the actin monomer binding protein, profilin which, when PIP_2-bound, may mediate the nucleation of actin (Wolven et al., 2000). Alternatively, PIP_2 targeted proteins may affect actin nucleation indirectly by interacting with other proteins such as the actin related protein (ARP2/3) complex (Yang et al., 2000). Results from a recent study demonstrate that PI-enriched plasma membrane rafts are preferred sites for actin polymerization events, which occur through a WASP-ARP2/3-dependent pathway in Swiss 3T3

fibroblasts (Rozelle et al., 2000). The authors demonstrated that PIP_2, PI kinases, and an unidentified tyrosine kinase localized in plasma membranes in a cholesterol-dependent manner and that all were essential for cytoskeletal reorganization. In addition, PIP_2 is capable of activating N-WASP *in vitro* (Rohatgi et al., 1999;Rohatgi et al., 2000), a protein recognized for its ability to promote the nucleation of actin (Taunton,). Because membrane-associated F-actin polymerization events are involved in a vast array of cellular activities, including vesicular trafficking (Taunton,), endocytosis (Lechler et al., 2000), focal adhesion (Ward and Hammer, 1993), synapse formation (Nielander et al., 1997), and the assembly of intercellular junctions (Wiebe et al., 2000), the functional implications of such microdomains are numerous. One such example is found in migrating adenocarcinoma cells, in which the establishment of front and rear polarity is cholesterol-dependent (Manes et al., 1999). In these cells, rafts preferentially localize to the leading edge and may drive cellular locomotion by affecting F-actin polymerization.

The Cortical Cytoskeleton

The membrane skeleton, also known as the cell cortex (Bretscher, 1991), is located beneath the plasma membrane and is comprised of a complex, three-dimensional molecular network formed by interacting cytosolic, peripheral, and transmembrane proteins (Bray et al., 1986). The core of membrane skeletal structure is composed of heterodimeric proteins, known variously as spectrin (erythroid-specific) or fodrin (non-erythroid eukaryotes), self-associate to form a polytetrameric backbone and provides the general framework of cortical structure (Waugh, 1983;Bretscher, 1991). The peripheral component of cortical structure, which includes F-actin and low molecular weight actin

binding proteins, interacts with plasma membrane proteins (Bretscher, 1991) and phospholipids (Goldmann et al., 1999), in highly dynamic fashion, forming the hallmark lipid-protein and protein-protein associations that inseparably link the structure of plasma membranes to that of the submembranous cytoskeleton and from which all eukaryotic species derive properties requisite for cell viability, the maintenance of cell shape, and signal transfer (Waugh, 1983;Noegel and Schleicher, 2000).

Mechanical Roles. The resiliency of plasma membranes to otherwise lethal structural stress is attributable to the submembranous cytoskeleton (Waugh, 1983) (Fig.1). A direct consequence of the intricate communication between the cortex and the plasma membrane is the coincident formation of cytoskeleton-based structures, such as focal adhesion complexes (Nikolopoulos and Turner, 2000) and adherens junctions (Richard et al., 1999;Takakuwa et al., 2000). These subcellular structures comprise dense submembranous networks of actin filaments and actin-binding proteins that sterically inhibit the movements of laterally diffusing of membrane proteins (Duband et al., 1988). Another cytoskeleton-based barrier effect, known as “cytoskeletal fencing” (Tsuji and Ohnishi, 1986), which results primarily from associations between the plasma membrane and the membrane skeleton, may promote either the aggregation or the segregation of membrane proteins and, thereby, affect protein function (Sheetz, 1993). Such structural domains may also provide a means to localize membrane proteins into topical areas overlying specific subcellular regions that in turn enable eukaryotes to compartmentalize and diversify their response to external stimuli (Jugloff and Jongstra-Bilen,

1997;Tsakiridis et al., 1999;Hurley and Misra, 2000;Sheng and Pak, 2000). Accordingly, the membrane skeleton may mediate and/or optimize the transduction of cell signals.

Signaling Functions. Just as the static portions of membrane skeletal structureimpart structural integrity to plasma membranes, the dynamic aspects of cortical structure are implicated in the transmission of cell signals (Bretscher, 1991;Aderem, 1992). Presently, widespread evidence obtained from a diverse array of studies exploring the cell biology of protozoans (Carbajal et al., 1996), yeasts (Field and Kellogg, 1999), plant cells (Vissenberg et al., 2000), and mammalian cells (Mullins, 2000) universally link the cell cortex to the local and global propagation of cellular signals, suggesting the cytoskeleton to be an integral part of eukaryotic signaling pathways. Of relevance to the perspectives presented in this dissertation is evidence that implicates the involvement of the membrane skeleton in the transmission of signals from activated transmembrane proteins, including chemoattractant receptors. That the membrane skeletal network is capable of directly interacting with activated chemoattractant receptors in neutrophils has been established (Jesaitis et al., 1993). Consistent with this finding, several studies have correlated the inhibition of F-actin polymerization with the inhibition or potentiation of downstream effector activities, including chemotaxis (Malech et al., 1977), degranulation (Mocsai et al., 1999), superoxide production (Jesaitis et al., 1986), and cell-substratum adhesion (Garnotel et al., 1995). Of these studies, those which link receptor signaling activity to physical associations of receptor and cytoskeleton are of interest. The possibility that such interaction will result in receptor-immobilization (Jesaitis et al., 1984) and/or the lateral displacement of receptors into unique structural domains (Jesaitis

et al., 1989;Jesaitis et al., 1993) have clear implications. While the former will affect the translational diffusion properties of receptors within plasma membranes, the latter event may accompany or immediately precede receptor trafficking events that modulate receptor-activation state (Jesaitis et al., 1989). Of the dynamic and diverse forces inherent in membrane structure, protein-protein, protein-lipid, and lipid-lipid interactions are especially relevant to the distribution, mobility, and regulation of cell-surface FPR. These interactions frequently either occur or are facilitated by the organelle fusion events triggered by neutrophil activation (Seely et al., 2003;Mahmudi-Azer and van Eeden, 2003). This in turn suggests that the physiological makeup of neutrophils contributes to not only the efficiency and regulation neutrophil activation but also the compartmentalization of hallmark signaling and effector activities that facilitate all aspects of neutrophil microbical function, including cellular chemotaxis, adhesion, and phagocytosis. Therefore, in order to further explore the cellular and molecular mechanisms that regulate neutrophil activation and cellular function, the processes implicated in maintaining the latent phenotype of nascent, circulating blood-neutrophil are next discussed.

Neutrophil Physiology

Neutrophils house an impressive diversity of organelles that are destined for secretion in a manner dependent on receptor engagement, cellular activation state, and ambient environmental factors, such as temperature (Bertram and Coignoul, 1982;Tzeng et al., 1984;Richter et al., 1989;Levitz and Farrell, 1990). The most well defined

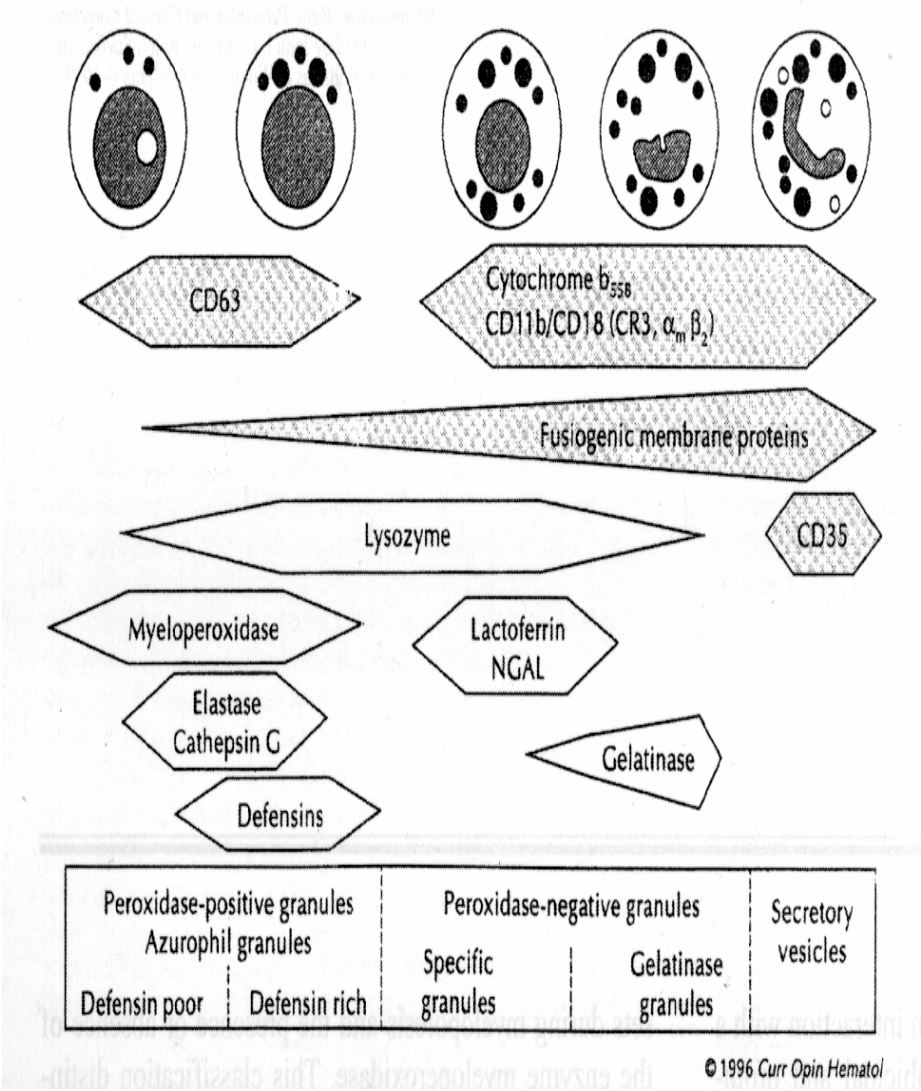


Figure 8. The progressive changes in neutrophil physiology occurring during development in the bone marrow. Note that the heterogeneity in composition between various organelles is likely to be developmentally related. The well-described, temporally distinct fusion events of these endosomal and granular organelles with plasma membranes confer distinct phenotypes to neutrophils. Such states facilitate cellular chemotaxis and cytotoxicity and support the existence of a graded neutrophil activation state. From Borregaard et al (Borregaard, 1996).

organelles are the primary (azurophilic) and secondary (specific) granules, which are named according to the order of their appearance during myelopoiesis within the bone marrow [(Bainton et al., 1971); (Figure 8)].

Primary granules are formed during pro-myelocyte stage of neutrophil development and are derived from the cis-Golgi, while secondary granules form during the myelocyte and meta-myelocyte stages and bud from the trans-Golgi. These granules comprise the bulk of the neutrophil microbicidal arsenal, with myeloperoxidase, acid hydrolase, and neutral protease activities primarily confined to the former and the bulk of antimicrobial peptides, transmembrane-cytokine, -complement, and –fMLF receptors, and NADPH oxidase components confined to the latter (Borregaard, 1996). Gelatinase is differentially expressed during the myelocyte and meta-myelocytes stages, and this provides a basis for the sub-classification of secondary granules [(Kjeldsen et al., 1994); (Figure 8)]. Thus, a third granule, referred to as tertiary granules, is formed during the late meta-myelocyte/early band stage of neutrophil development (Bainton, 1999). Tertiary granules are enriched in the specific granule protein, gelatinase, and are therefore considered a subset of secondary granules. Additional peroxidase-enriched granules that are distinct from the primary granules have also been identified (Parmley et al., 1987). The secretory fraction is comprises another vesicle subset that contributes to the microbical arsenal of neutrophils but is distinguished from the major granule subsets described above on the basis of its morphology (Kobayashi and Robinson, 1991), endocytic origin (Borregaard et al., 1990), and ability to affect and phenotypically define

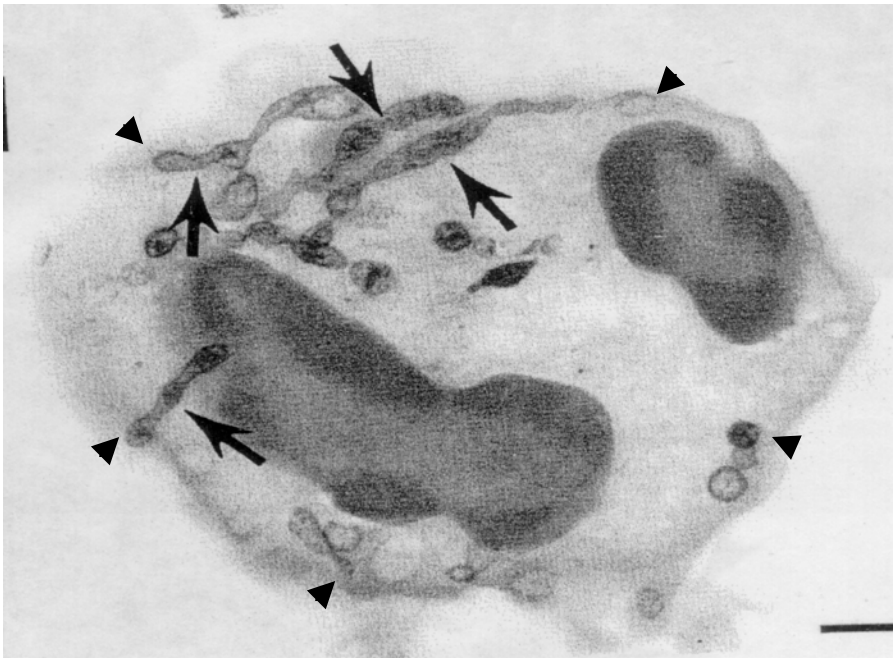
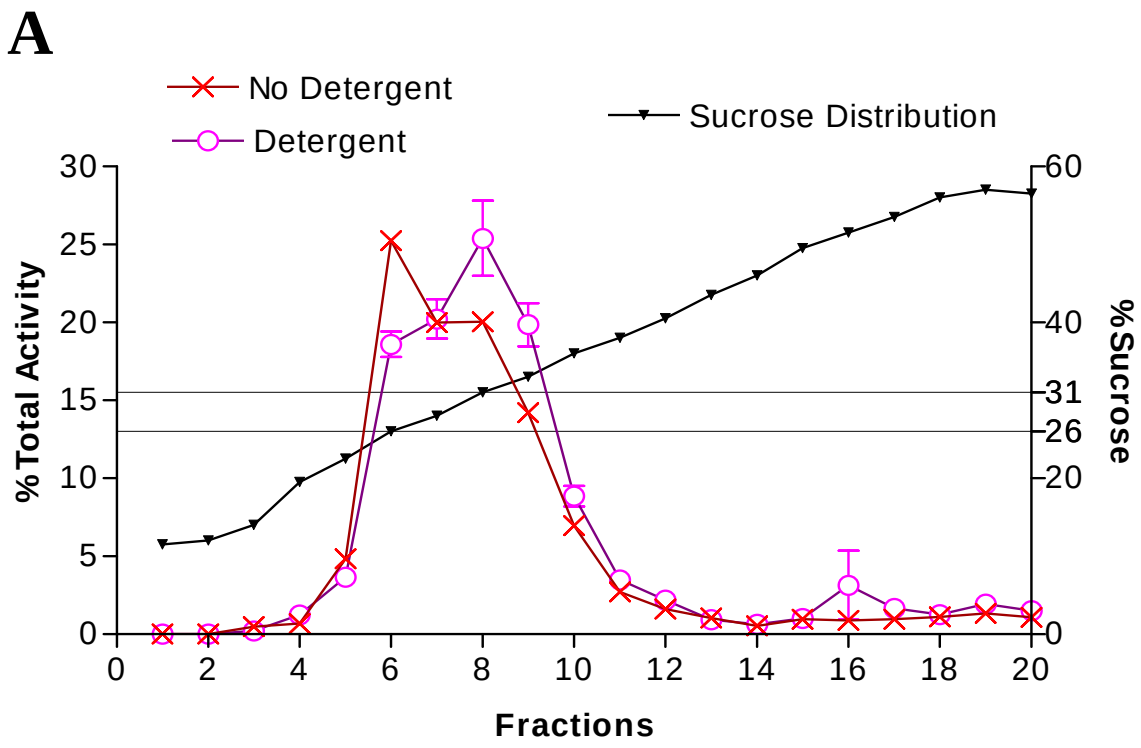


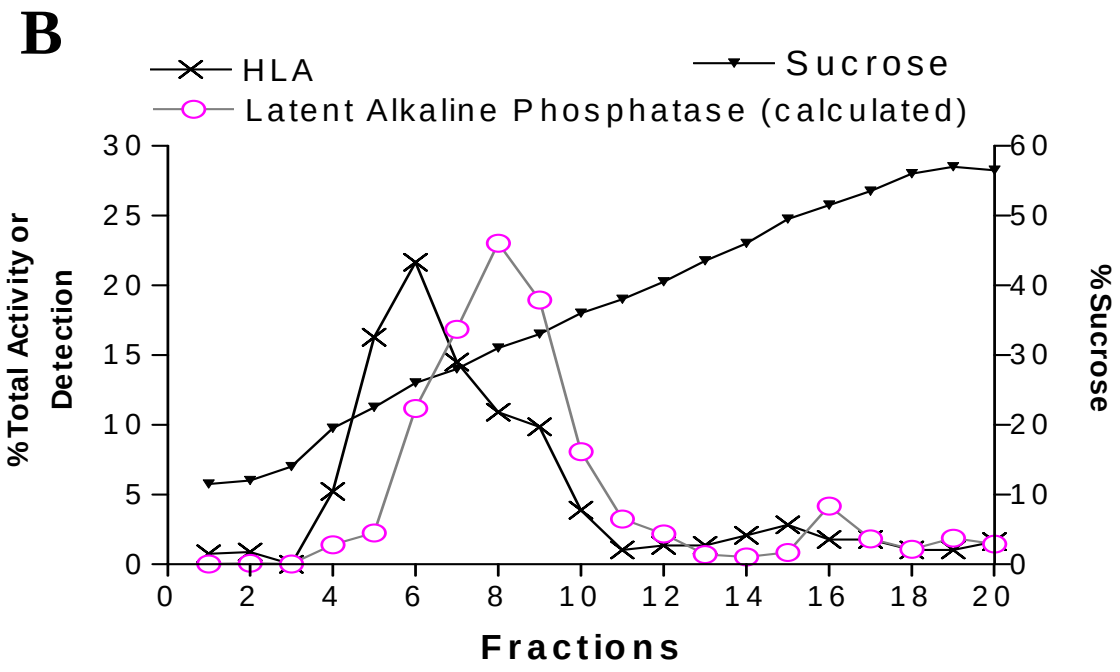
Figure 9. Morphologic features of the human neutrophil secretory organelle. The morphology of the secretory fraction is depicted in the partially activated phenotype of suspended neutrophils following uniform exposure to fMLF for 5 minutes. Note that secretory-plasma membrane fusion events occurs preferentially within defined regions of the plasma membrane. Thus, this mobilization event is associated with significant changes in plasma membrane morphology. Such fusion events may occur preferentially at the leading edge of the cellular migration and facilitate the incorporation of "activated," PML-enriched membrane into extending pseudopods (see text). Arrows point to secretory membranes at the cell surface that retain their morphologic identity due to the partial fusion of this organelle with plasma membranes. Arrowheads indicate discreet regions of secretory-plasma membrane fusion. From Robinson et al. (Robinson et al., 1999).

the ‘primed’ state of cellular activation (Borregaard et al., 1987);(Sengelov et al., 1993a;Borregaard et al., 1994). Secretory vesicles house a number of antimicrobial integral membrane proteins, including the FPR(Sengelov et al., 1994a), complement receptors (Sengelov et al., 1994b), and NADPH oxidase components (Sengelov et al., 1992), that are rapidly upregulated to plasma membranes where they contribute to the specialized primed phenotype of activated neutrophils.

One of the most profound attributes of this organelle is its shape. Unlike most other vesicles of endocytic origin, the secretory vesicles are tubular in shape (Fig 9). Prior to their mobilization to activated plasma membranes, secretory vesicles undergo a global reorganization that involves a series of homotypic, end-to-end fusion events and the formation of long tubular structures (Robinson et al., 1999). Although these structures subsequently fuse with plasma membranes, they maintain their tubular morphology and are not entirely integrated into plasma membrane bilayer structure, suggesting that the maintenance of this shape is linked in some way to the cellular activation state and/or effector function (Figure 9).

The kinetics of secretory vesicle mobilization relative to the mechanics of cellular movement is unknown. However, it is thought that its partial integration within post-fusion, primed plasma membranes may provide a reserve of activated membranes. This reserve is enriched in FPR as well as other chemoattractant receptors and signaling molecules, that can be utilized for signal transduction and pseudopod extension. This possibility is supported by observations made by light microscopy of chemotactically migrating neutrophils. In such cells, activated plasma membrane-bound chemoattractant


A
No Detergent
Detergent
Sucrose Distribution
% Total Activity
% Sucrose
Fractions
0
5
10
15
20
25
30
0
20
26
31
40
60
0
2
4
6
8
10
12
14
16
18
20


B
HLA
Latent Alkaline Phosphatase (calculated)
Sucrose
% Total Activity or Detection
% Sucrose
Fractions
0
5
10
15
20
25
30
0
10
20
30
40
50
60
0
2
4
6
8
10
12
14
16
18
20

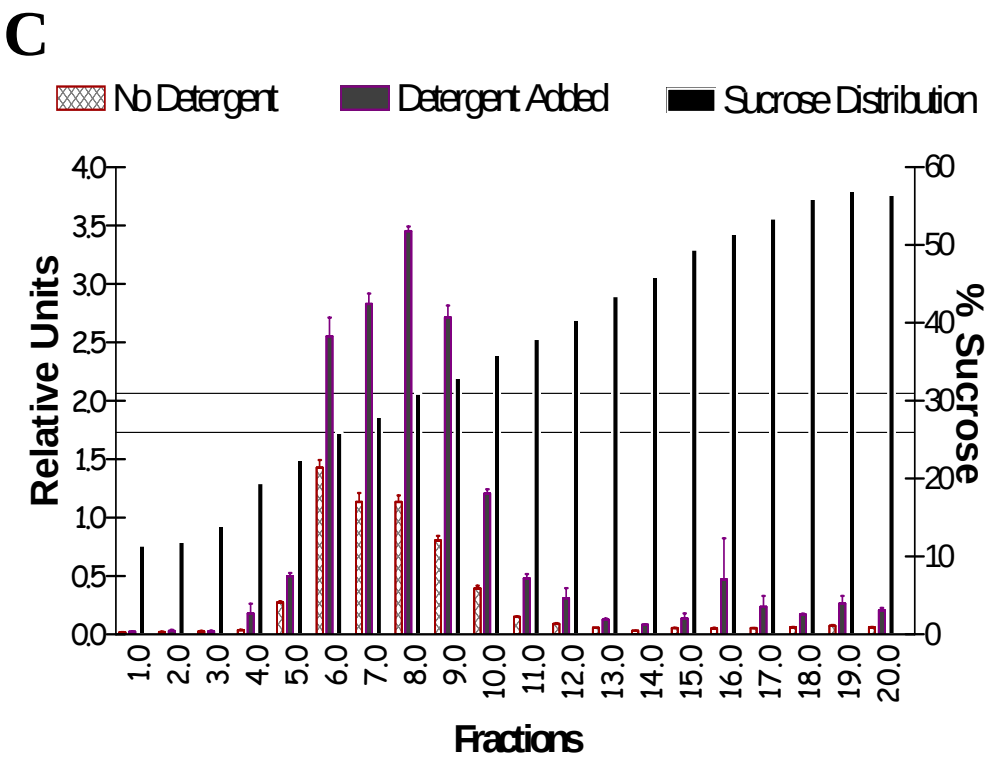


Figure 10. Differential subcellular localization of alkaline phosphatase activity in human neutrophils isolated by preparative methods that either preserve or deplete populations of the secretory vesicle organelle. Immediately after isolation, populations were prepared for analysis by N_2 cavitation and isopycnic sucrose density sedimentation. **A**) Analysis of detergent-free (x) versus total cellular (○) alkaline phosphatase activity in gradient fractions from nonactivated neutrophil populations isolated by a dextran-based method. Both activities are calculated as percent total activity. However, for comparison, detergent-free alkaline phosphatase activity was calculated relative to cumulative detergent-free activity. Activity in the presence of detergent is bimodal in distribution with peaks at 29% and 33% sucrose. The peak plasma membrane activity is indicated by detergent-free alkaline phosphatase with a single peak at 29% sucrose. By comparison, the bimodal distribution of enzyme activity in the presence of detergent indicates that alkaline phosphatase is sequestered in vesicles of slightly higher density (33% sucrose) than activity obtained in the absence of detergent (28% sucrose). **B**) The intravesicular alkaline phosphatase activity suggested in (A) was calculated as 'latent' activity by measuring the difference in enzymatic activity obtained in the presence and absence of detergent for each gradient fraction. The peak latent activity (○) is of slightly higher density than the peak activity obtained for the plasma membrane marker, HLA (x). The calculated curve is derived from the data shown in (A). **C**) The distribution of alkaline phosphatase activity obtained in the presence and absence of detergent is compared for each gradient fraction. Detergent-free activity is shown as a percentage of total cellular

(Figure 10 –continued) activity on the y-axis, and thus suggests that the larger fraction of cellular alkaline phosphatase activity is sequestered in neutrophils prepared by the dextran method. Note that the enzymatic activity obtained in the absence of detergent (striated column) was fully retained in presence of detergent (filled column) and indicates that the shift in enzymatic activity is valid. The relatively thinner, solid black columns represent % sucrose (right axis). The two fraction shift in enzymatic activity (fraction 6 to fraction 8) confirm both the presence and subcellular distribution of the 'latent' alkaline phosphatase distribution shown in B. Data from figures A, B, and C are from six separate experiments. Little variation was obtained between experiments, as noted. Data are from unpublished studies of JS and AJJ.

receptors appear to maintain a random distribution except for the transient, periodic accumulation of receptors associated with the pseudopodial membrane. Such transient accumulation of receptor has been attributed to the targeted exocytosis of chemoattractantreceptor-enriched granule subpopulations to the pseudopodial membrane (Sullivan et al., 1984;Walter and Marasco, 1984;Servant et al., 1999). Interestingly, neutrophil granule subpopulations have been shown to target the membranes of developing phagosomes during their upregulation to the cell surface (N'Diaye et al., 1998;Perskvist et al., 2002;Cougoule et al., 2002;Vieira et al., 2003), suggesting that vesicle fusion events are indeed spatially-directed.

Investigations seeking to identify precisely when in the neutrophil lifecycle secretory vesicles are formed are ongoing. However, evidence suggests that formation occurs shortly after maturation (Borregaard, 1996). Although derived from plasma membranes, this fraction does not recycle within mature neutrophils but instead comprises a stable endosomal pool in circulating, quiescent cells (Borregaard et al., 1987). This organelle has been formerly defined as a secretory compartment due to the presence of tetranectin, an 82 kDa protein that is typically expressed by and secreted from secretory cells (Borregaard et al., 1990). Intact secretory vesicles, furthermore,

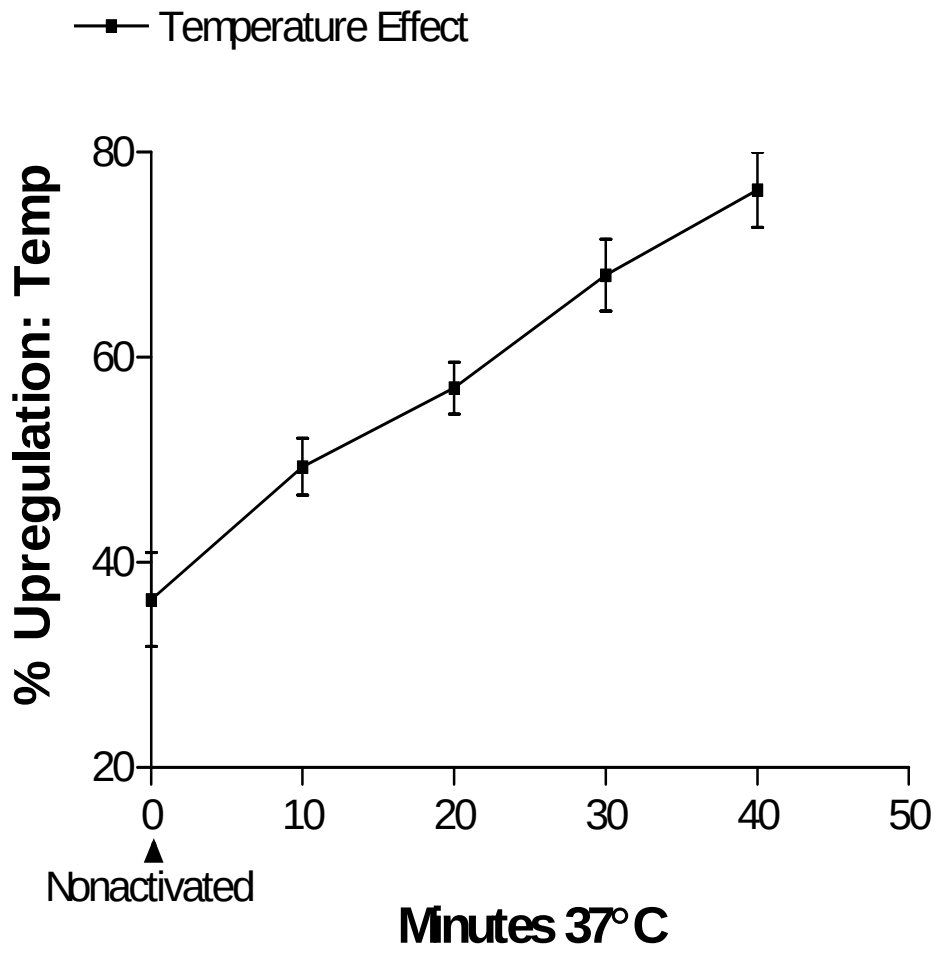


Figure 11. Temperature-dependent, spontaneous exocytosis of secretory vesicles. Secretory vesicles are stable under *in vivo* conditions within circulating neutrophils. Once neutrophils are purified from whole blood, however, secretory vesicles destabilize and are spontaneously lost over time. The data show secretory vesicle loss over time at 37°C in the absence of agonist. Secretory vesicles were quantitated by assessing latent alkaline phosphatase activity (Kobayashi et al., 1998a). Data are from unpublished studies of J.S. and A.J.J.

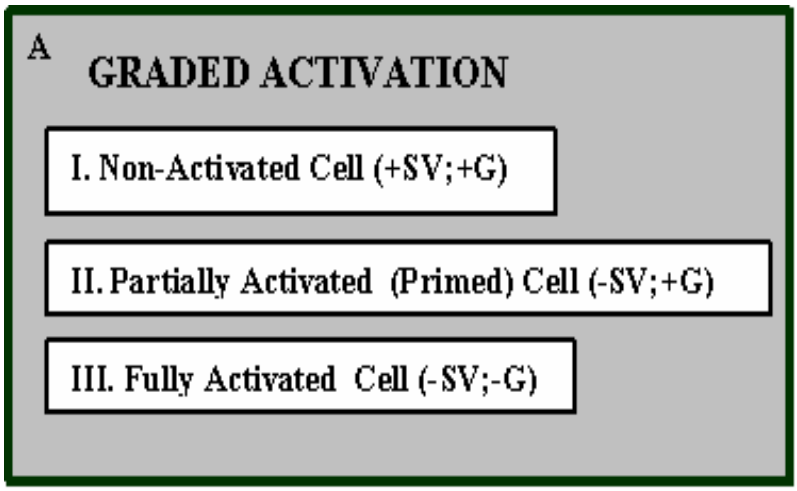
contain most of the cellular alkaline phosphatase activity (Borregaard et al., 1987), as well as a variety of receptors and effector molecules important for extravasation (Borregaard et al., 1994), chemotaxis (Sengelov et al., 1994a), and microbicidal function (Bjerrum and Borregaard, 1989). Thus, mobilization of this compartment to plasma membranes may confer a partially activated, or primed, cellular state (Borregaard et al., 1987). Although stable in circulating, quiescent cells, this fraction may be rapidly lost in response to perturbations experienced during the process of neutrophil isolation from whole blood (Figure 10) or by subjecting *in vitro* isolated neutrophils to environmental changes, such as temperature modification [(Borregaard et al., 1987); Figure 11)]. This latter issue is addressed in greater detail in Chapter 2, which uses different techniques for blood-neutrophil isolation, such as dextran- and gelatin-based methods, to examine the various aspects of secretory vesicle loss, including:

(i) The relative content of intact secretory vesicles in human neutrophils isolated by dextran-based and gelatin-based preparatory methods.

(ii) The integration of exocytosed secretory vesicle membranes in existing plasma membrane structure.

(iii) Procedural considerations of the gelatin-based technique that significantly affect the extent of secretory vesicle loss in gelatin-purified neutrophil populations.

The Physiological Significance of the Secretory Fraction. Mobilization of secretory organelles is rapidly and organelles is rapidly and universally inducible following the exposure of neutrophils to



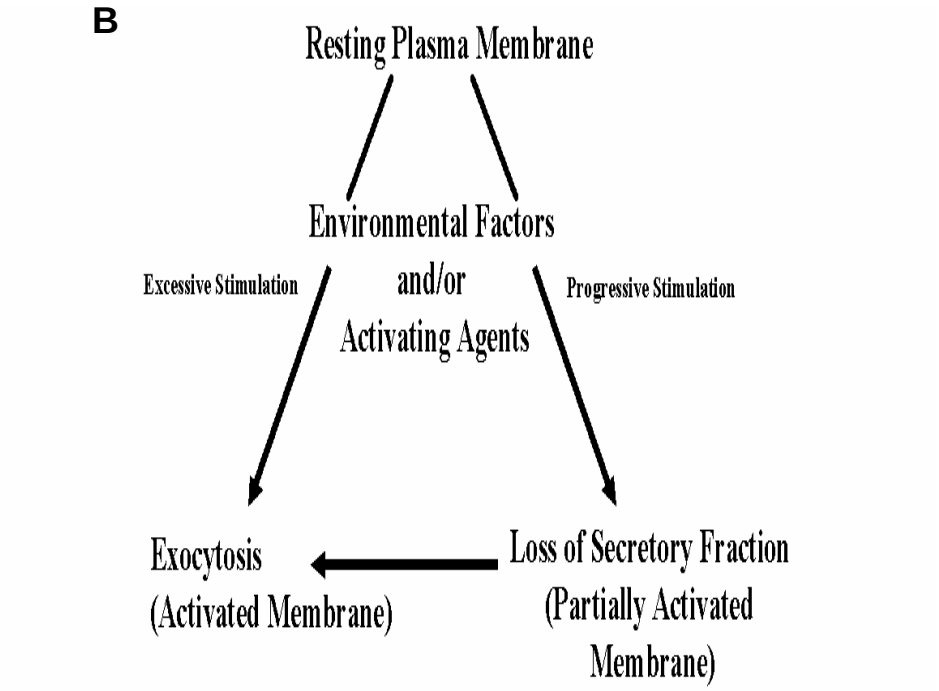


Figure 12. Progressive nature of human neutrophil activation. Neutrophil activation is inherently graded. **A**) Non-activated cells (I) become partially activated (II) following secretory vesicle (SV) -plasma membrane fusion events but retain their primary and specific granule (G) complement (-SV;+G). Partially activated, or primed, cells may proceed to fully activated stage (III) and, thus, lose their primary and specific granule fraction (-SV;-G). **B**) The activation kinetics of human neutrophils can be potentiated

(Figure 12 –continued) depending on the nature and quantity of stimuli cells encounter. For example, when neutrophils encounter either multiple or a single agonist in high concentration. Under such conditions, the excytosis of secretory vesicles occurs coincidently with that of tertiary granules and the partial exocytosis of specific granules. Alternatively, under conditions where stimulation is gradual and progressive, the release of secretory vesicle and granule subpopulations occurs concomitantly.

activating ligands *in vitro* and thus essential for the phenotypic conversion from a non-activated, or resting, cellular state to one capable of mediating directed migration and antimicrobial functions (Sengelov et al., 1993a;Borregaard et al., 1994;Borregaard et al., 1995). Thus, the fusion of secretory and plasma membrane organelles is considered the foremost event preceding neutrophil activation. The kinetics of its upregulation support this view, as the secretory fraction is the most rapidly mobilized of neutrophil organelles (Borregaard et al., 1990). It follows, therefore, that the loss of the secretory fraction appears to be requisite for cellular activation (Borregaard et al., 1995) and implies that the conversion from resting to fully activated states is inherently graded (Figure 12). This graded course of cellular activation likewise results in a graded phenotype, as primed cells are capable of extravasation and chemotaxis but deficient in cytotoxicity (Yasui et al., 1994;Gewirtz and Simons, 1997), and fully activated cells encompass the full spectrum of neutrophil effector function (Turner et al., 1994).

Several ultrastructural, cytochemical, and biochemical studies have examined the functional consequences of secretory vesicle exocytosis in either phorbol myristic acid (PMA)-stimulated or fMLF-stimulated neutrophils and, in particular, correlated the kinetics of secretory vesicle upregulation with the induction of specific microbicidal functions. Such studies have found that the neutrophil transition from a nonactivated state to one that is actively antimicrobial is preceded by and dependent upon secretory vesicle-

plasma membrane fusion events. Accordingly, when cytochemical and ultrastructural methods were employed to trace the origin of NADPH-oxidase activity in fMLF-stimulated cells, the reaction product was found exclusively associated with intracellular secretory vesicles and, to a lesser extent, specific granules (Kobayashi et al., 1998a;Seguchi and Kobayashi, 2002). Further, the kinetics of secretory-plasma membrane fusion events correlated with the biochemical detection of superoxide release following initial stimulation (Kobayashi et al., 1998a). Remarkably, these data suggest that the components of the NADPH-oxidase system are not recruited to plasma membranes but to secretory vesicles. It is thus the post-fusion secretory vesicle membranes that confer the capacity of superoxide generation on plasma membranes. It follows, therefore, that, first, the fusion secretory-plasma membrane compartments results in the delivery of biochemical pathways to the cell surface that are otherwise absent in resting plasma membranes and, second, that these two organelles are innately and differentially associated with distinct signaling effectors. It would thus appear that acquisition of effector phenotype is dependent upon the rapid upregulation of secretory vesicles to resting plasma membranes (Borregaard et al., 1987;Sengelov et al., 1993b;Borregaard et al., 1994). Consistent with this concept, the mobilization of secretory organelles occurs by a mechanism that is biochemically distinct from the well-described exocytic pathway for primary and secondary granules.

In addition to being $G\alpha_i$-dependent, secretory vesicle traffic is distinctly calcium-independent (Kobayashi et al., 1998b). These observations support the concept that surface-upregulation of secretory organelles both precedes and facilitates directed

migration, given that the initial mechanical events of chemotaxis are calcium independent and that the kinetics of upregulation parallel the activation of effector protein function in fMLF stimulated cells (Kobayashi et al., 1998a;Kobayashi et al., 1998b;Kobayashi et al., 2000). Moreover, other analogous studies have confirmed secretory vesicle-plasma membrane fusion events to both precede and to be required for chemotaxis (Murakami et al., 1998;Kobayashi et al., 2000). Accordingly, the efficacy of FPR engagement in previously nonactivated plasma membranes to induce the functional conversion of neutrophils may be attributable to the extent secretory endosomes are upregulated to cell surfaces (Kobayashi et al., 1998a;Kobayashi et al., 1998b). Biochemical and/or morphological testing of resting neutrophils in the above-cited studies, furthermore, ruled out the possibility of artifactual results due to the assessment mixed (nonactivated and pre-activated) populations. This confirmation insures an accurate, unambiguous assessment of the activation properties of resting membranes in these investigations. As such, these studies significantly contribute to a conceptual basis for understanding the physiologic importance of secretory vesicle exocytosis in neutrophil activation.

The molecular or underlying basis for the markedly different functional properties that characterize resting and activated plasma membranes remains unknown. One hypothesis is that the plasma membranes of activated human neutrophils, which lack secretory vesicles, possess a distinct microdomain structure that supports a signaling capacity that is either deficient or altogether absent in the plasma membranes of resting neutrophils (Mukherjee et al., 1994). The study described in Chapter 3 specifically

addresses this issue by correlating the selective exocytosis of secretory vesicles with (i) significant changes in the microdomain structure of neutrophil plasma membranes and (ii) the functional responsiveness of intact neutrophil populations to fMLF stimulation, in terms of superoxide generation.

Biology and Physiology of FPR Activation and Signal Transduction

Plasma membranes provide a specialized organizational and biochemical framework for the transmission of receptor-mediated signals (Barnett-Norris et al., 2005;Lin and Shaw, 2005;Becher and McIlhinney, 2005;He et al., 2005). Many fundamental defense functions, including cellular chemotaxis, are transduced within specialized lipid and/or membrane-cortical microenvironments (Kwiatkowska et al., 2003;Pizzo and Viola, 2004). Recent biochemical and functional studies suggest that plasma membrane lipid heterogeneity contributes to vital aspects of chemoattractant receptor function in immunocytes, including the generation, propagation, and compartmentalization of signal transmission (Manes et al., 1999;Gomez-Mouton et al., 2001). Directed migration to formylated peptides, a major class of chemoattractants released by infecting bacteria, is mediated by the FPR, a member of heptahelical GPCR superfamily (Yang et al., 2001a). Importantly, the FPR exhibits organizational regulation within plasma membranes.

The FPR is selectively expressed in neutrophils and facilitates their rapid accumulation at extravascular sites of infection (Boulay et al., 1990). The molecular basis of FPR activation and desensitization is temporally and metabolically coupled to the

lateral redistribution of occupied receptors into compositionally distinct functional compartments that differ in relative density (Jesaitis et al., 1988;Jesaitis et al., 1989;Jesaitis et al., 1993;Jesaitis and Klotz, 1993;Klotz and Jesaitis, 1994a;Klotz and Jesaitis, 1994b). Indeed, major model systems depicting mechanistic aspects of FPR regulation emphasize that receptor activity is influenced by a number of systems functioning at the lipid matrix and membrane cortical levels (Jesaitis and Klotz, 1993;Servant et al., 2000;Key et al., 2003). These model systems, however, are incomplete, with each presenting distinct or overlapping scenarios of receptor signaling and/or processing. Thus, critical pieces of information pertaining, in particular, to potential molecular associations of FPR with cytosolic, structural, and membrane-bound regulatory molecules still need elucidation.

Our current understanding of FPR function is that occupied FPR is subject to a number of redundant feedback regulatory processes that may involve allosteric changes mediated by receptor associations with cortical actin, membrane-bound, and soluble proteins. Such dynamic, ligand-dependent alterations in receptor conformation may thus provide a molecular cue for the lateral segregation of receptors into functional compartments within the plane of the membrane as well as provide internalization cues, or directly inactivate the receptor.

This section will review current concepts in FPR biology and physiology. Emphasis will be on the role of the local environment of the bilayer in which the formyl peptide receptor (FPR) resides, its native distribution pattern, and properties intrinsic to the receptor itself. It is assumed that agonist-binding may induce or promote subtle

alterations in receptor conformation that feedback and modify the receptor-activation state, affect receptor-mobility and thus collisional interactions in plasma membranes, and intrinsic receptor signaling capacity.

Local Environment of the FPR. The FPR is predicted to span the plasma membrane seven times. From an extracellular perspective, taking the rhodopsin structure as a model for heptahelical receptors, the first through seventh transmembrane segments arrange counter-clockwise to give the receptor an oval or bean-shaped morphology (Quehenberger et al., 1993). Given the extensive transmembrane aspect of the FPR and its somewhat compact conformation in the bilayer, FPR activity may be subject to changes in the local composition of membrane phospholipids. Although evidence supporting this possibility for FPR is indirect, the importance of membrane composition in directing the activation and/or regulation of other seven transmembrane chemoattractant receptors is substantial. Interestingly, activated FPR promotes lipid signaling activity that is:

i) Colocalized to select regions of the plasma membrane bearing activated heptahelical receptors (Servant et al., 2000).

ii) Capable of modifying the traffic of membrane-associated signaling molecules downstream of activated receptors and affecting the translocation of low molecular weight proteins from the cytosol in response to receptor activation (Liscovitch et al., 1994;Blanc et al., 2005;Diaz-Valencia et al., 2005).

iii) Responsible for signal amplification at the leading edge of the cell membrane during chemotaxis (Parent and Devreotes, 1999;Servant et al., 2000).

iv) Confined to membrane domains of defined lipid composition, e.g., membrane rafts (see above), where regulated phospholipid metabolism occurs (Hashizume et al., 1996;Matecki and Pawelczyk, 1997;Janes et al., 1999;Singh et al., 2001;Becker and Hannun, 2005).

It is thus plausible that changes in the phospholipid composition of the bilayer either enhance or limit the extent of FPR-activation and mobility. Further support for this concept is provided by the observation that heterotrimeric G proteins partition into Triton X-100 detergent-insoluble glycosphingolipid enriched complexes (DIGs). Such lateral compartmentalization of receptors suggests that certain heptahelical receptors, upon activation, localize to topologically distinct regions of the bilayer (Li et al., 1995;Ostrom et al., 2000). The critical and widespread role of such domains in regulating related receptors strongly supports the importance of membrane lipid heterogeneity in the initiation of signaling cascades by agonist-bound FPR. In addition, it is possible that the native FPR, in the absence of activating ligand, preferentially colocalizes to membranous regions devoid of downstream signaling molecules in order to prevent the inappropriate activation of FPR. This possibility is apparent in view of the high constitutive activity of FPR in heterologous expression systems (Wenzel-Seifert et al., 1998a). Either scenario would couple FPR-activation with a nonrandom organization of receptor in plasma membranes. Evidence from previous studies suggests that native (nonactivated) and

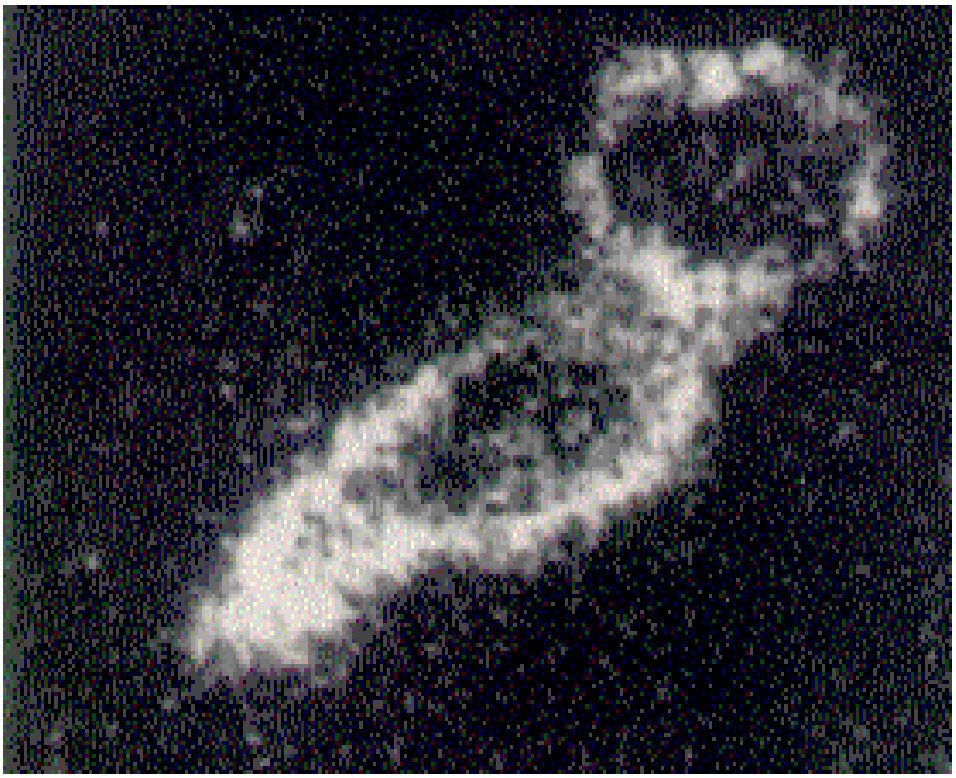


Figure 13. FPR distribution in resting (nonactivated) human neutrophil plasma membranes. Presented above is a confocal image from Johansson et al. demonstrating the specific labeling of glass adherent neutrophils with the antagonist tert-butyloxycarbonyl-Phe(D)-Leu-Phe(D)-Leu-Phe-OH. Because the binding of this ligand to the FPR does not elicit FPR activity, this distribution is representative of native surface receptor. This figure is from reference (Johansson et al., 1993).

activated FPR are differentially distributed into compositionally distinct lateral domains of neutrophil plasma membranes (Jesaitis et al., 1988;Jesaitis et al., 1989).

Native Distribution Pattern of the FPR. Native, antagonist-labeled, FPR appear uniformly distributed within the intact plasma membranes of viable, human neutrophils,as viewed with confocal microscopy [(Johansson et al., 1993); Figure 13)]. These receptors are divided into freely diffusible and immobile receptor populations. According to other studies, the distribution of activated chemoattractant receptors were not significantly altered during fMLF-or C5a-induced neutrophil chemotaxis (Servant et al., 1999;Servant et al., 2000). On the other hand, the regional confinement of membrane receptors may lead to their heterogeneous distribution and, in some cases, to their aggregation (Niedel, 1981;Hofer et al., 1998). A random, unclustered, homogeneous distribution of a freely diffusible membrane protein might suggest that a surface receptors are mostly free from intermolecular associations that might hinder or otherwise restrict their free diffusion within plasma membranes (Sheetz, 1993). Nonetheless, a substantial fraction of the FPR (45%) present in purified, unstimulated neutrophil membranes is found associated with the membrane skeleton, anchoring the plasma membrane (Klotz et al., 1994). Several possibilities that may adequately account for this observation include:

i) Associations with other proteins anchored to the actin cytoskeletal framework.

ii) Direct associations with actin

iii) Associations with lipids anchored to the cytoskeleton.

These possibilities derive from properties intrinsic to the receptor itself. In addition, changes in cellular environment inevitably occur during and subsequent to the purification of neutrophils from donor blood. The introduction of exogenous contaminants or the release of proteins from dead and/or dying cells might promote cellular changes that could lead to nonphysiological responses. Specific receptor-cytoskeleton associations or the nonspecific entanglement of receptor within inducible, dynamically formed membrane-bound cytoskeletal networks may occur in response to changes in membrane phospholipid composition prompted by modifications in pH, temperature, and/or membrane structural stress introduced by the sedimentation process (Godin and Garnett, 1976;Marinetti and Love, 1976).

Intrinsic Properties of the FPR. Unlike some enzymatic transmembrane receptors that are intrinsically capable of transducing signals immediately following ligand recognition, it is generally recognized that FPR-activation is two-dimensional, requiring recognition of external ligand in concert with the lateral coupling of membrane-bound heterotrimeric G proteins (Stadel et al., 1980). The FPR may, however, be precoupled with G proteins in the absence of ligand (Domalewski et al., 1996). In the presence of guanosine 5'-triphosphates, the agonist-bound, G protein-coupled receptor complex initiates multiple signaling cascades that drive the key cellular processes from which neutrophils derive their trademark defensive function (Torres and Ye, 1996). For example, FPR-activation promotes cell locomotion along shallow molecular gradients of formylated peptide released by bacteria resident at sites of infection (Perez et al., 1991), as well as the production of microbicidal superoxide (Cederholm and Gyllenhammar,

1999). Given the potential toxicity associated with FPR activation (Hashida et al., 2000), random, agonist independent receptor activation could lead to the inappropriate release of cytotoxic compounds. Consequently, strict cellular control over inappropriate, or ligand-independent, receptor activity would be expected.

The family of G protein coupled receptors (GPCRs) to which the FPR belongs is highly heterogeneous in terms of function (Ulloa-Aguirre et al., 1999). Most GPCR members demonstrate some degree of basal activity (Costa et al., 1992). Proposed models describing the thermodynamic (Onaran et al., 1993) and allosteric (Stadel et al., 1980) bases for the formation of ternary complexes predict that this activity is intrinsic to all GPCRs. Generally, however, chemoattractant receptors are not measurably active in the absence of ligand (Wenzel-Seifert et al., 1998b). In light of this, the FPR presents an intriguing dilemma. Recent evidence reveals that FPR expressed in SF9 insect cells co-expressing G protein possess considerable basal activity (Wenzel-Seifert et al., 1998b). Assuming that FPR expression in insect cells accurately reflects the activation state of the FPR expressed in mammalian expression systems, this newfound property may account for several previous findings. Accordingly, in one study, a considerable fraction of immobilized receptor was found to be present in the plasma membranes of viable, unstimulated neutrophils (Johansson et al., 1993). Similarly, another group of investigators found that a significant portion of the FPR in purified plasma membranes obtained from unstimulated neutrophils co-sedimented with the membrane skeleton in Triton-X-100 insoluble pellets (Klotz et al., 1994). In fact, high levels of basal activity may predispose a significant fraction of native receptor toward constitutive associations

with the cytoskeleton, which could account for the above findings. One implication is that constitutively active receptors would assume a conformation that is similar oridentical to that associated with the agonist-bound state. Such changes in the receptor activation state might result from the steady state coupling of native receptor with G protein. This would indicate that, in the absence of agonist, the conformation of native FPR in neutrophil plasma membranes is not static and that changes in receptor conformation may result in agonist-independent changes in receptor-cytoskeleton interaction. Such interaction may also follow agonist-dependent receptor-G protein activation and thus be regulatory in nature. In any case, there are many unanswered questions about the significance of native receptor-cytoskeleton associations and the modulatory capacity of receptor-cytoskeletal interactions especially in terms of relative changes in the activation and distribution of receptors.

The functional and molecular nature of FPR-cytoskeleton association is remarkably heterogeneous, in that FPR-membrane skeleton associations appear to mediate multiple effector outcomes. Three well-documented effects that characterize FPR-cytoskeleton association in fMLF-treated neutrophils are the stabilization of receptor-ligand complexes into high affinity complexes (Jesaitis et al., 1984), receptor desensitization (Jesaitis et al., 1989), and the pre-endocytic, physical sequestration of agonist-bound receptors into distinct, preexisting membrane domains (Jesaitis et al., 1989;Jesaitis et al., 1993). Significantly, these effects, all of which signify key aspects of FPR traffic within neutrophil plasma membranes, can be inhibited by pretreatment with dihydrocytochalasin B (dhCB), a compound that disrupts microfilament structure (Jesaitis

et al., 1985). There is also evidence indicating that FPR-cytoskeleton associations are not purely regulatory in nature, and, further, that the FPR is capable of interacting with distinct species of F-actin. For example, certain FPR-cytoskeleton associations that correlate with the regulation of downstream effector activities are inhibited in dhCB-treated, fMLF-stimulated neutrophils (Jesaitis et al., 1985); on the other hand, dhCB-treatment has no detectable effect on constitutive FPR-membrane skeletal associations that occur in the absence of receptor activation and/or neutrophil activation (Klotz et al., 1994). Both types of interactions, dhCB sensitive and dhCB insensitive, are actin-based, as indicated by the disruption of the latter when membranes obtained from unstimulated neutrophils are (i) solubilized in the presence of other known actin-severing agents, including p-chloromercuriphenylsulfonic acid and DNase I, or (ii) subjected to conditions that promote F-actin disassembly, such as solubilization in the presence of elevated concentrations of KCl (Klotz et al., 1994).

The differential sensitivity of FPR-cytoskeletal complexes to dhCB confirms previous results obtained in separate studies suggesting the existence of distinct cellular pools of F-actin in a wide array of eukaryotic cell-types (Goodlad and Clark, 1993;Olorundare et al., 1993;Patterson et al., 1993;Thiele and Steinbach, 1994;Zegers and Hoekstra, 1998;Zegers et al., 1998;Tzima et al., 2000). The formation of detergent insoluble native receptor-cytoskeleton complexes and the F-actin-mediated down-regulation of activated surface FPR supports the notion that the FPR can interact with distinct pools of F-actin (Klotz et al., 1994). Indeed, that key effector activities downstream of the FPR appear to be regulated exclusively by the formation of dhCB sensitive FPR-cytoskeleton complexes

suggests an intriguing scenario wherein the selective interaction of receptor with disparate species of F-actin potentially mediates multiple but independent effector outcomes. To date, however, the functional nature of dhCB-insensitive FPR-cytoskeleton associations has not been investigated in detail.

FPR-Mediated Signal Transduction and Regulated Secretory Vesicle Exocytosis. Formylated peptides (e.g., fMLF), released by invading bacteria at extravascular sites of microbial infection, serve as physiological ligands for the FPR (Gao et al., 1994). Ligand recognition is followed by a diverse and formidable signaling cascade that is transduced by the differential engagement of effector molecules by heterotrimeric G protein components, α and βγ, and results in the activation of phospholipases C (PLC;(Verghese et al., 1986)), PLD (Ortmeyer and Mohsenin, 1993;Djerdjouri et al., 1999), and PLA_2 (Cockcroft and Stutchfield, 1989;Syrbu et al., 1999;Sternfeld et al., 2000;Marshall et al., 2000); protein kinases of the SRC family (Ptasznik et al., 1995;Welch and Maridonneau-Parini, 1997), protein kinase C (Pike et al., 1986); small molecular weight G proteins of the Rho and Ras family (Zheng et al., 1997;Fensome et al., 1998;Wong et al., 2006); phosphoinositide kinases, PI4P-5K and PI-3K (Ptasznik et al., 1995;Sternfeld et al., 2000;Yamamori et al., 2004;Condliffe et al., 2005), and mitogen activated kinases, including extracellular regulated kinases-1 and -2 (Rane et al., 1997;Fujita et al., 2005;Selvatici et al., 2006). Thus, one key consideration in FPR signal transduction is the organization of effectors in nonactivated and activated plasma membranes. In addition to the above, the finding that FPR can couple with multiple isoforms of G alpha, which can in turn differentially

engage unique signaling pathways [(McLeish et al., 1989;Bokoch, 1995); Figure 12], emphasizes the likelihood that plasma membranes are inherently ordered. For example, in one experimental system, the differential segregation of serpentine cAMP receptors in plasma membranes may result in the selective and differential coupling of receptors to functionally distinct effector pathways requisite for directed movement (Xiao and Devreotes, 1997;van Haastert and Kuwayama, 1997;Jin et al., 2000;Moniakis et al., 2001). Similarly, the FPR pools present in resting plasma membranes and secretory organelles could be coupled to different signaling pathways, which in turn implies that short and long-range effector capacity of FPR varies with the cellular state of activation. Evidence for this contention rests in:

i) The correlation of FPR activation to upregulation of secretory organelles (Borregaard et al., 1992;Sengelov et al., 1993a;Sengelov et al., 1993b;Borregaard et al., 1994;Sengelov et al., 1994a;Kobayashi et al., 1998a;Kobayashi et al., 2000;Nagano et al., 2001);

ii) Kinetics and interdependence of upregulation relative to downstream effector function (Borregaard et al., 1987;Kobayashi et al., 1998a;Kobayashi et al., 1998b;Kobayashi et al., 2000;Seguchi and Kobayashi, 2002).

Thus, various studies examining the mechanistic bases of drug-induced effects on neutrophil function have correlated initial FPR engagement with the $G\alpha_i$ –and PIP_3 – dependent upregulation of secretory organelles to surface membranes (Nagano et al., 2001). This process occurs within a timeframe that coincides with the acquisition of

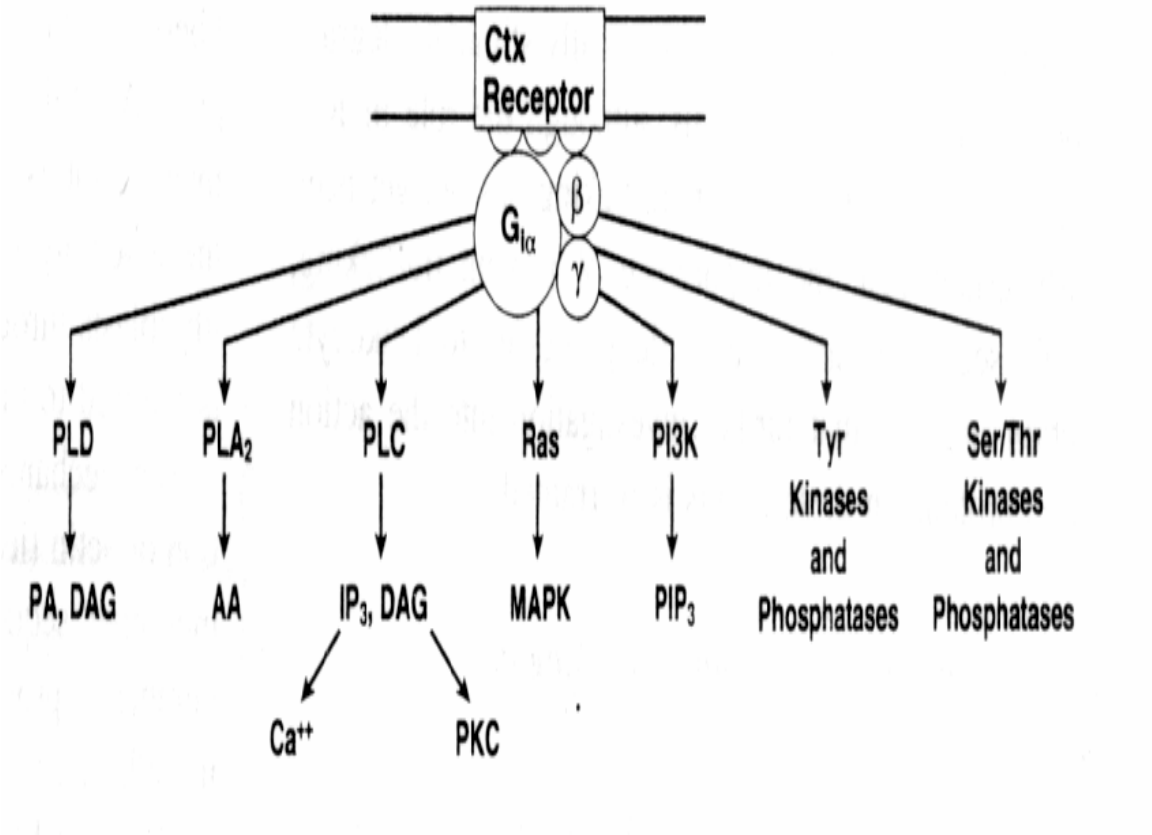


Figure 14. FPR signal transduction in human neutrophils. The diverse and formidable array and signaling effectors that interact differentially with G protein subunits, α and βγ, following the FPR activation. From G.M. Bokoch {39}.

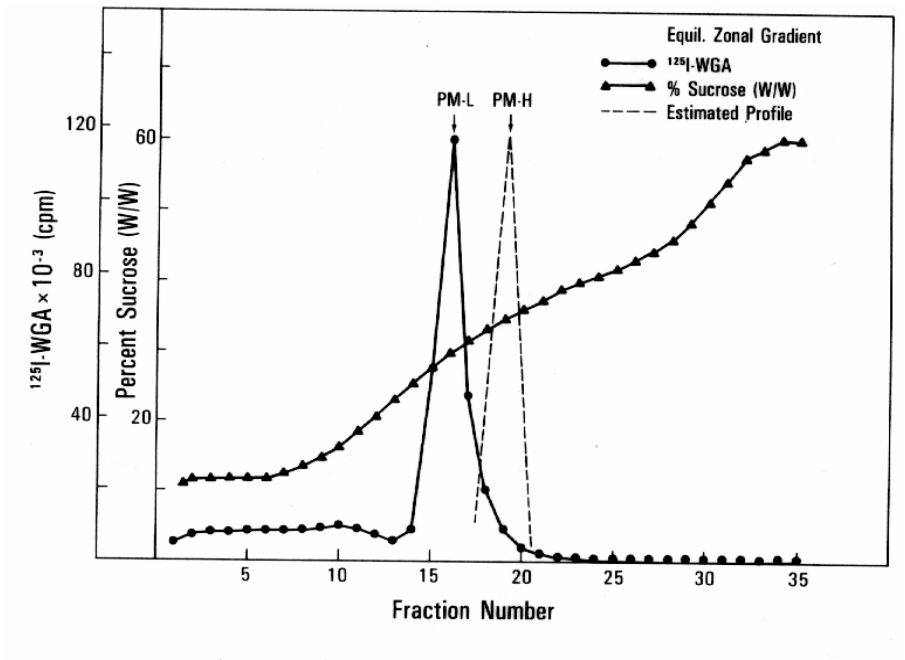
effector function (Kobayashi et al., 1998a;Kobayashi et al., 1998b;Kobayashi et al., 2000;Lavastre et al., 2002). Significantly, if secretory vesicle mobilization is impaired, for example, by pre-incubating cells with microtubule inhibitors (Kobayashi et al., 1998b), neutrophils do not activate, thus indicating that secretory membranes are indeed the activated membranes that either confer or facilitate the range of effector functions demonstrated by primed and fully activated neutrophils. Several studies have, furthermore, suggested that secretory vesicles and plasma membranes are differentially enriched in signaling molecules required for cellular chemotaxis (Kobayashi et al., 1998a;Seguchi and Kobayashi, 2002). Rac GTPase (Kobayashi et al., 1998a), PIP_2, and PIP_3 (Fig 14) are of particular interest in this regard because recent models directly attribute the mechanistic basis of directed migration in neutrophils to a closed, positive feedback loop involving these molecules (Benard et al., 1999;Chung et al., 2000). Indeed, indications that secretory organelles are differentially enriched in D_4 phosphoinositides and cytoplasmic signaling proteins, additionally implies the residence of specialized, highly potent signal transducing phospholipid microdomains within this fraction.

Given the large number of downstream effectors in FPR signal transduction and the markedly diverse array cellular responses that are possible, it is likely that spatial control over pathway assembly and effector activity is most probably an important aspect of signal regulation by neutrophil plasma membranes (Barabe et al., 2002a). Several plasma membrane-derived structures implicated in the organization of signaling effectors have been identified in human neutrophils, including rafts (Seveau et al., 2001a) and caveolae (Yan et al., 1996;Yu et al., 2000). Both are enriched in saturated

lipids, cholesterol (Simons and Ikonen, 1997), and, possibly, D_4 phosphoinositides, including PIP_2 and PIP_3 (Rozelle et al., 2000;Martin, 2001), and the clustering of these lipids induces discreet changes in the lipid phase of otherwise homogeneously disordered membranes that attract the partitioning of peripheral and transmembrane proteins (London and Brown, 2000;Meyer zum Bueschenfelde et al., 2004;Silvius, 2005;Jiao et al., 2005;DeBruin et al., 2005). The principal class of proteins found in domains of this kind are cytosolic effector molecules, primarily SRC kinases (Schade and Levine, 2002), small molecular weight G proteins (Niv et al., 2002), and G alpha subunits (Moffett et al., 2000), each post-translationally modified with one or more saturated acyl chains (Buss et al., 1987;Schultz et al., 1987). Although the membrane association of this class of proteins may be inducible or constitutive, once in the membrane they preferentially partition into the ordered lipid environment of membrane rafts and/or caveolae (Ilangumaran et al., 1999;Tooze et al., 2001). Rafts and caveolae differ morphologically, compositionally, and in terms of their relative mobility in plasma membranes. Caveolae can assume a variety of shapes, the most common being vesicular. This form is a result of their membrane association with caveolin, a hairpin-shaped, transmembrane protein that interacts with membrane cholesterol and the subcellular cortex, effectively immobilizing this domain-type in membranes (Anderson, 1998). Rafts, in contrast, are planar and highly mobile, so that their appearance in membranes would resemble a traditional flat, wooden raft floating on a river (Simons and Ikonen, 1997).

Of the two domains to which FPR localize in neutrophils (i.e., PMH or PML; see below), the plasma membrane light domain (PML) is likely related to one of the two

A



B

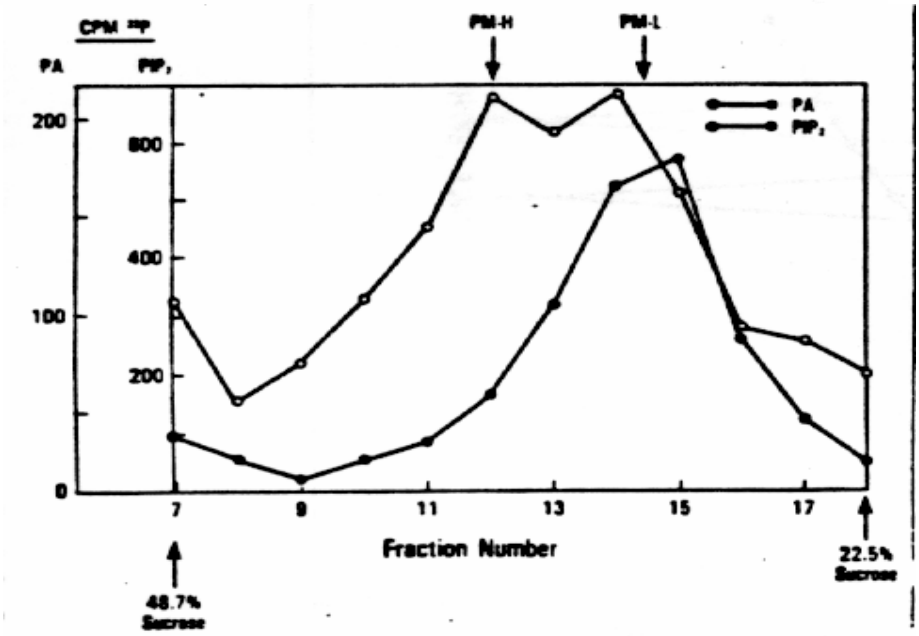


Figure 15. Differential lipid enrichment of the PML and PMH microdomains. Unpublished data of A..J. Jesaitis depicting the isolation (A) and partial characterization (B) of plasma membrane light and heavy domains (PML and PMH, respectively). Note the differential enrichment of the PML and PMH in phosphatidylinositol-4, 5-bisphosphate (PIP_2) and phosphatidic acid (PA), respectively.

specialized domains described above, as it is enriched in cholesterol and PIP_2 [(A.J. J, unpublished data; Figure 15)]. Further, since this domain is enriched in heterotrimeric Gα subunits (Jesaitis et al., 1989), it is possible that PML predominantly localizes to secretory organelles, given additional data indicating that secretory organelles are Gα subunit-enriched (J.S. and A.J.J, unpublished) and the exclusive localization of effector proteins to secretory vesicles during the course of their exocytosis [e.g., Rac GTPase (Kobayashi et al., 1998a)]. Significantly in this respect, PIP_2 and Rac GTPase are thought to be central effectors of neutrophil migration (Wu et al., 2000). In activated plasma membranes, regulated changes in phosphoinositide metabolism determine where and when Rac GTPase is activated by controlling the localization and activity of Rho family-directed guanine nucleotide exchange factors (Shinohara et al., 2002;Welch et al., 2002). The localized, activated GTP-associated Rac GTPase, in turn, physically associates with and activates phosphotidylinositol 4, 5-kinase, amplifying PIP_2 production (Tolias et al., 2000;Tolias and Carpenter, 2000). PIP_2 plays a crucial and versatile role in neutrophil activation and effector function, in particular, serving to modulate and organize signaling cascades by providing membrane attachment sites for pleckstrin domain-containing effector molecules (Harlan et al., 1994;Pitcher et al., 1995). Pleckstrin domain-containing proteins function both as exchange factors for certain low molecular weight GTPases (Terui et al., 1994;Brown et al., 2001) and as cofactors for key enzymatic activities (Liscovitch et al., 1994;Pertile et al., 1995;Whatmore et al., 1996). In addition to these functions, PIP_2 is also implicated as the primary regulator of the actin cytoskeleton during

chemotaxis (Janmey, 1994), facilitating spatially restricted actin polymerization processes by stimulating gelsolin to sever and uncap barbed (fast growing) filament ends. The mechanics of directed movement may accordingly be affected by Rac GTPase-regulation of PIP_2 production at the leading edge of migration (Tolias et al., 1998;Carpenter et al., 1999). It would seem, therefore, that the spatial regulation of signaling proteins is paramount for this effector function. Thus, it is clear that the multiplicity of signaling effectors that associate with phospholipid microdomains and suggest that these structures markedly increase signaling efficiency in a pathway-specific and physiologically relevant manner in neutrophils (Schreiber et al., 2000;Doughman et al., 2003;Golub et al., 2004;Golub and Caroni, 2005;Downes et al., 2005). Many of the effectors mediating directed motility in these cells colocalize with intact secretory vesicles of unprimed neutrophils. As PML and PMH microdomains are present only in primed versus nonactivated neutrophils (Chapter 3), we hypothesize that the delivery of these effectors to the PML compartment during initial stimulation is a central preparatory event for chemoattractant activation.

As demonstrated in Figure 15, both PML and PMH are enriched in the fusogenic glycerophospholipid, phosphatidic acid (PA), which directs intracellular fusion events (Lebrand et al., 2002). Significantly, the vectoral transport of secretory organelles to specialized regions of surface membrane has been proposed to occur (Peranen et al., 1996;Kobayashi et al., 1998a;Hattula et al., 2002;Hattula and Peranen, 2005). Accordingly, given that superoxide generation is a function of secretory vesicle membranes (McLeish et al., 1989;Kobayashi et al., 1998a;Seguchi and Kobayashi, 2002),

its targeted delivery to developing phagosomes is a physiological imperative, as this would confer microbicidal activity where it is most needed while minimizing damage to host tissues (Vissers et al., 1985;Johansson et al., 1995). These data indirectly support the physiologic importance of secretory vesicle upregulation in FPR function by indicating that fusogenic secretory vesicle-plasma membrane interactions in primed and fully activated neutrophils are typified by a highly directed and differential interaction that localizes activated, PML-enriched membrane at the leading front of cellular migration. Because the lipid and protein composition of the PML and PMH remains largely uncharacterized, their relationship to rafts and caveolae and, hence, their relative signaling capacities, are uncertain. However, given the marked relevance of secretory-plasma membrane organelle fusion to phenotypic conversion (Sengelov et al., 1993a;Borregaard et al., 1994), detailed compositional analysis of the PML/PMH microdomains is important for understanding a potential role for receptor segregation FPR-mediated neutrophil chemotaxis. This in turn could help broaden the mechanistic and molecular bases of cellular migration to one that includes selective, inter-organelle cooperative interactions. Such interactions would be fundamental to the regulation of receptors in resting plasma membranes and to the inducible re-organization of plasma membranes following receptor activation. One possible implication of such dynamic, subcellular reorganization of secretory endosomes is that it reflects a cooperative process facilitating the targeted delivery of activated membrane to cell surfaces in response to specific signals generated by FPR-ligand complexes.

FPR Regulation and the Cellular Activation State. Members of the superfamily, to which FPR belongs, are subject to diverse forms of regulation in activated plasma membranes. These can be broadly categorized as associated with one or more of the following processes.

i) Direct structural modification, such as by phosphorylation (Key et al., 2001), selective and inducible lipid associations (Pyne and Pyne, 2002), or receptor dimerization (Jordan et al., 2001).

ii) Spatial segregation by the confinement of surface receptors within regions of defined lipid or protein composition (Jesaitis et al., 1988); such regions, classically referred to as microdomains, may be derived from plasma membrane structure (Rybin et al., 2000) and/or cortical structure (Jesaitis et al., 1989) and are often constitutively present in resting plasma membranes (Jesaitis et al., 1989).

Temporal regulation, which involves both the removal (Bunemann and Hosey, 1999) and delivery (Thivierge et al., 1999) of receptors from/to activated plasma membranes; while the latter can result in a rapid increase in the number of surface receptors by the regulated delivery of internal pools of receptor. The former is often involved in the desensitization of activated receptors. The FPR is subject to all three forms of regulation described above, including phosphorylation (Key et al., 2001), differential segregation (Jesaitis et al., 1988;Jesaitis et al., 1989), and temporal up- and down regulation by delivery (Sengelov et al., 1994a) to or removal (Yang et al., 2001b)

from activated membranes. Of these regulatory processes, the conditional segregation of surface receptors into pre-formed microdomains appears to be a principle mechanism of FPR regulation in human neutrophils (Jesaitis and Klotz, 1993;Klotz and Jesaitis, 1994a). Accordingly, non-activated receptors occupy domains of light-density (PML), which are enriched in G protein alpha subunits and possibly other downstream components involved in FPR signal transduction (Jesaitis et al., 1988). Following activation, receptor-desensitization occurs by receptor-translocation into domains of heavier density (PMH) that are enriched in various cytoskeletal components, including actin and fodrin, but devoid of G protein subunits (Jesaitis et al., 1989).

The Compartmentalization of FPR in Plasma Membranes

While the relative importance of the different mechanisms of FPR regulation in neutrophils remain unclear, several lines of evidence emphasize the importance of plasma membrane domain structure in all critical aspects of FPR activity, including facilitation of G protein coupling (Jesaitis et al., 1988), downstream signal transfer and/or effector activity (Servant et al., 2000), and receptor desensitization processes (Jesaitis et al., 1986;Jesaitis et al., 1993). In contrast to the well-described function of GRK/Arrestin function in the regulation of many GPCRs, the role of these classical regulators in the biology of neutrophil FPR desensitization (Key et al., 2003), endocytosis (Gilbert et al., 2001), and recycling (Vines et al., 2003) remains to be defined. This section will focus on the role of membrane structure in FPR activation and discuss the relevance of classical desensitization pathways in FPR downregulation.

Compartmentalization and Signaling Efficiency. Membrane-derived, structurally distinct supramolecular complexes appear to be involved in FPR activation and desensitization processes. These membrane complexes include cholesterol-enriched lipid (Frasch et al., 2004) and membrane-cortical assemblies (Jesaitis et al., 1988;Seveau et al., 2001a;Eddy et al., 2002). Recent studies have emphasized the critical importance of plasma membrane cholesterol content in the fMLF-induced polarization and migration of primary, adherent cells. These investigations are consistent with previous results demonstrating a primary role for membrane organization in the modulation of FPR function (Jesaitis et al., 1986;Jesaitis et al., 1988;Jesaitis et al., 1993). Specifically, plasma membrane associated FPR are dynamically and conditionally compartmentalized into compositionally and functionally distinct lateral domains that differ in relative density. However, unoccupied receptors may be either freely diffusible or immobilized within intact resting neutrophil plasma membranes (Johansson et al., 1993). Once occupied, receptors segregate into effector-enriched membrane domains (i.e., PML; ρ = 1.12 g/cc), which likely facilitate the kinetics, signaling specificity, and focal transduction of signals necessary for the onset of polarization and directed migration (Jesaitis et al., 1988;Jesaitis and Klotz, 1993). Receptor-catalyzed GTP/GDP exchange on the G-alpha subunit of the associated heterotrimeric G proteins require specific, conformational changes of FPR that in turn facilitate further regulatory interactions. Among such interactions are those with the actin component of the membrane skeleton, which locks receptors into a high affinity state that prevents receptor-G protein interactions (Jesaitis et al., 1993). Receptor-actin complexation, thus, desensitizes

receptors and induces a simultaneous lateral shift into effector-depleted, fodrin/actin-enriched domains, where receptors are subsequently internalized (Jesaitis et al., 1989). Indeed, the fact that PML and PMH are present only in primed and activated human neutrophil plasma membranes (see Chapter 3) emphasizes the functional nature of these compartments. Moreover, findings concerning the dominant role of plasma membrane structure and organization in FPR regulation agree with numerous other studies examining receptor regulation of functionally diverse GPCR subtypes, including classical beta-2 adrenergic (Williams et al., 1995;Xiao et al., 1999;Schwencke et al., 1999)and rhodopson (Polozova and Litman, 2000;Deretic et al., 2004) model systems, in which receptors conditionally and differentially translocate into and out of compositionally distinct membrane compartments, in response to agonist interaction.

Additional insight into the physiological importance of the compartmentalized transduction of FPR-generated signals is provided by recent findings demonstrating that receptor activation leads to the plasma membrane-presentation of select, granule-specific antimicrobial activities. In this instance, plasma membrane localization is facilitated by the preferential interaction of microbicidal proteins with activated neutrophil plasma membranes following granule exocytosis. In contrast to the prototypical function of cellular microdomains in organizing pathway-specific responses to environmental stimuli, this type of extracellular presentation may represent an additional, novel extension of the concept of plasma membrane compartmentalization in activated, degranulated human neutrophils. The functional significance of such interactions presumably concerns the localization microbicidal proteins at extracellular sites on

plasma membranes where phagocyte-microbial interactions occur (Boxer et al., 1982;Petrequin et al., 1986;Perez et al., 1989;Afeltra et al., 1997;Kettritz et al., 1997;Manev et al., 1998;Kocher et al., 1998;Tse et al., 1999;Deriy et al., 2000;Tse et al., 2000;Xiao et al., 2002;Tanaka et al., 2003;Reumaux et al., 2003;Caccavo et al., 2003;Schreiber et al., 2004;Xiao et al., 2005). For example, the high affinity interaction of the specific granule protein, lactoferrin, with the plasma membranes of activated neutrophils both facilitates its delivery into developing phagosomes and enhances the rate and efficiency of phagocytic activity (Manev et al., 1998). Similarly, the study presented in Chapter 4 of this dissertation elucidates the capacity another specific granule protein, hCap, to bind surface membranes of activated (partially or fully degranulated) human neutrophils with high affinity. The functional significance of this interaction is suggested by the absence of hCap expression on the plasma membranes of resting (non-activated) neutrophils, as well as the significant enhancement of surface expression in response to progressive changes in the neutrophil activation state.

FPR-Arrestin Interactions. Whereas studies in primary cells have stressed the critical importance of plasma membrane organization in FPR signaling and regulation, studies employing heterologous expression systems have elucidated the potential regulatory importance of FPR-G protein receptor kinase (GRK) interactions. These studies have shown that occupied FPR are phosphorylated on two distinct sets of serine/threonine enriched motifs encompassing residues 328-332 and 334-339, located within the carboxy terminal domain of receptor (Maestes et al., 1999). Although phosphorylation of serine residues within one motif (325-334) was found to promote

arrestin-binding (Key et al., 2003), phosphorylation of either motif alone, in the absence of arrestin, was sufficient to induce desensitization through steric uncoupling of FPR-G protein interactions (Bennett et al., 2001) and receptor-internalization (Maestes et al., 1999). Further, FPR endocytosis proceeds through an arrestin/dynamin/clathrin-independent mechanism, and FPR-dependent MAPK activation is independent of receptor internalization (Gilbert et al., 2001). Interestingly, however, FPR-arrestin interactions parallel the regulatory effects of FPR-actin interactions by locking occupied receptors into high affinity G protein-poor complexes (Jesaitis et al., 1993;Key et al., 2001). Similarly, FPR phosphorylation, by sterically uncoupling FPR-G protein interactions (Bennett et al., 2001), also appear to mimic the functional effects of FPR-actin interactions by preventing receptor-G protein interactions. Thus receptor phosphorylation and receptor-arrestin associations may act in conjunction or in parallel with cortical F-actin to expedite segregation of occupied FPR into G protein poor lateral domains.

The FPR and Signaling Scaffolds

One intriguing aspect of FPR signaling potential is the possibility of FPR-scaffolding activities that permit G protein-independent signaling cascades. Many functionally diverse GPCR subtypes have this capacity, which is either encoded within the primary amino acid sequence of receptors or conferred through ligand-induced conformational changes in receptor-tertiary structure (Hall et al., 1999;Premont and Hall, 2002;Brady and Limbird, 2002). As many as 50 cytosolic molecules, consisting of adaptor, signaling, transcription, and cytoskeleton proteins have been identified to

specifically associate with various GPCRs in an inducible manner, and many of these interactions have been functionally characterized (Bockaert et al., 2003).

Given the complexity and sophistication of FPR activation and regulatory processes and the significant gaps in our knowledge concerning the basic biology of FPR processing, the potential for such ancillary receptor associations appears promising. For example, a growing number of studies examining the G protein-independent signaling potential of functionally diverse GPCR subtypes indicate that intracellular domains of GPCR structure can nucleate formation of biologically relevant multi-molecular signaling complexes. Specific associations, direct and indirect, between membrane receptors and various cytosolic intermediates are a common, defining feature in the activity of other receptor families, including integrins (Geiger and Bershadsky, 2001;Bershadsky et al., 2003) and tyrosine kinases (Davy et al., 2000b;Ridyard and Robbins, 2003). Such interactions regulate behavioral aspects of specialized cell types, for example, through temporal and spatial organization of various cellular activities, including the hallmark processes of focal adhesion complex formation in adherent fibroblasts (Zimerman et al., 2004) and immunosynapse (Hiltbold et al., 2003) development between interacting immunocytes.

The Physiological Importance of GPCR Scaffolds. The function of G protein-independent GPCR signaling has been demonstrated in several experimental systems, including mammalian neuronal cells (Kreienkamp, 2002;El Far and Betz, 2002) and dictyostelium (Brzostowski and Kimmel, 2001). These interactions culminate in the assembly of condensed GPCR-multiprotein units that preserve integrity of signal transfer,

for example, by an exclusion-based minimization of crosstalk between different receptors and the pathways they activate (Mahon et al., 2002). Biological significance of such complexes is far-reaching and includes modulation of GPCR- trafficking (Sheng and Kim, 2002) and subcellular targeting (Sheng and Kim, 2000), in addition to specific signaling activities (Hall, 2004). Many non-G protein-GPCR interactions involve motif sequences enriched in hydrophobic and/or acidic residues within the GPCR C-terminus that are recognized by PDZ domains (Sheng and Sala, 2001). Significantly, of the three classes of functional motifs identified thus far, FPR C-terminal residues house a candidate class II motif, i.e., φ-x-φ, where φ are hydrophobic residues and x denotes any amino acid. The physiologic importance of such interactions is illustrated in cultured cerebellar granule neurons, wherein differential association of G_0-coupled metabotropic glutamate receptor (mGluR) subtypes with protein interacting with C kinase 1 (PICK-1), through a class II motif present in mGluR7a (El Far et al., 2000;Dev et al., 2001) but absent in mGluR2 (Perroy et al., 2001), provides a primary GPCR-based mechanism for ligand-dependent modulation of electrochemical homeostasis . Such highly refined, subtle differences in the activity of related GPCR subtypes, suggests that other GPCR-mediated cellular processes may be modulated by similar interactions. Recently, a scaffolding protein, Hem-1, expressed in human neutrophils has been found to regulate neutrophil polarity and chemotaxis by organizing macromolecular assemblies in developing pseudopodial membranes that insure compartmentalized assembly/activity the actin cytoskeleton, through localization of Rac-GTPase-WAVE-2 complexes, as well as a positive feedback circuit involving phosphatidylinositol-(3,4,5)-tris-phosphate, Rac-

GTPase, and F-actin that is essential for the genesis and maintenance of neutrophil polarity (Weiner et al., 2006). Furthermore, CXCR4-directed migration was found to involve direct functional associations between the carboxy terminal domain of receptors and components of the myosin II complex (Rey et al., 2002). These data suggest that other aspects of GPCR signaling, including transduction of chemotaxis, are governed by receptor-scaffolding activities.

Significance of GPCR Scaffolds in Chemotaxis. Many specific, functionally defined G protein-independent GPCR interactions are functionally relevant to chemotaxis (Bockaert et al., 2003). Such associations encompass a diverse array of cytoskeletal and signaling proteins, including myosin heavy chain IIA, fodrin, actin, protein 4.1, CapZ, filamin, and calmodulin (Hjalm et al., 2001;Rey et al., 2002;Becamel et al., 2002;Enz, 2002). It is of great interest that many of these proteins facilitate cell migration and, moreover, are directly implicated in the physical sequestration of chemoattractant receptors in different experimental systems. In addition, adaptor proteins that serve as dedicated scaffolding proteins are now directly implicated in:

i) The generation and spatial and temporal regulation of the amplified, phosphatidylinositol-(3,4,5)-tris-phosphate and Rac-GTPase-mediated signaling gradients that underlie polarity development in neutrophils (Weiner et al., 2006);

ii) The segregation of front-and-back signals in polarized leukocytes (Innocenti et al., 2004;Weiner et al., 2006);

iii) Maintaining of functional and morphological asymmetry of pseudopod and uropod structures in chemotaxing cells (Shinohara et al., 2002;Weiner et al., 2006).

Indeed, direct interactions between myosin heavy chain IIA and chemokine receptors CCR5 and CXCR4 in T lymphocytes (Rey et al., 2002) suggests a mechanism by which receptors become functionally segregated in plasma membranes. Available evidence, thus, supports the conclusion that GPCR signaling potential is remarkably extensive, involving a number of diverse protein families.

Molecular Mechanisms of Leukocyte Chemotaxis

Leukocyte chemotaxis has been intensively investigated, and much is known regarding the mechanics of cellular movement and biochemical progression of cell signaling that culminates in directed migration. In general, cellular movement is mediated by the actin-enriched cell cortex, which is in turn regulated by signals transmitted downstream of agonist-bound chemoattractant receptors (Katanaev, 2001). This section will review molecular events in leukocytes that underlie cellular chemotaxis.

Heterotrimeric G protein-Mediated Signaling Cascades.

The cell signaling cascades responsible for chemotaxis are derived from both α and βγ-mediated effector engagement (Clapham and Neer, 1997). The earliest events include βγ-activation of phosphoinositide-dependent PLC and phosphotidylinositol 3-kinase (PI-3K; (Belisle and Abo, 2000;Siddiqui and English, 2000)). The release of calcium from PLC activation and diacyl glycerol production activates a diverse array of serine-threonine kinases, including diacylglycerol-

dependent PKC. PI-3K facilitates the activation of small, low molecular weight G proteins of the Rho (Weiner et al., 2002), Rac (Belisle and Abo, 2000), and Ras (Knall et al., 1997) families by facilitating the docking and the localized activation of guanyl nucleotide exchange on distinct guanine nucleotide exchange proteins. Low molecular weight G proteins are also instrumental in the calcium-independent mobilization of secretory endosomes to plasma membranes (Nagano et al., 2001), which may additionally occur by a calcium and microtubule-dependent mechanism.

Role of PLD in Chemoattractant Receptor and Microbicidal Signaling. PLD hydrolyzes choline-containing phospholipids to produce phosphatidic acid and free choline [(Hanahan and Chaikoff, 1947;Hanahan and Chaikoff, 1948); (Figure 16)]. In human neutrophils, PLD signaling contributes to the regulation of a number of microbicidal activities, including phagocytosis (Suchard et al., 1997;Lennartz, 1999;Iyer et al., 2004;Lee et al., 2004;Corrotte et al., 2006), secretion (Kanaho et al., 1991;Nakamura et al., 1994;Mansfield et al., 2002;Mansfield et al., 2004), and production of superoxide anions by the NADPH oxidase complex (Suchard et al., 1994;Balazovich et al., 1996;Erickson et al., 1999;Kaldi et al., 2002;Mansfield et al., 2002). Two mammalian genes expressing PLD have been identified, including PLD1 and PLD2 (Frohman et al., 1999;Sung et al., 1999a;Sung et al., 1999b), with only the PLD1 gene product detected in human neutrophils (Marcil et al., 1997). A selective screening approach based on a bimolecular reconstitution of purified proteins in the absence of cytosol identified the ADP-ribosylation factor (ARF) GTPase as a potent activator of PLD activity

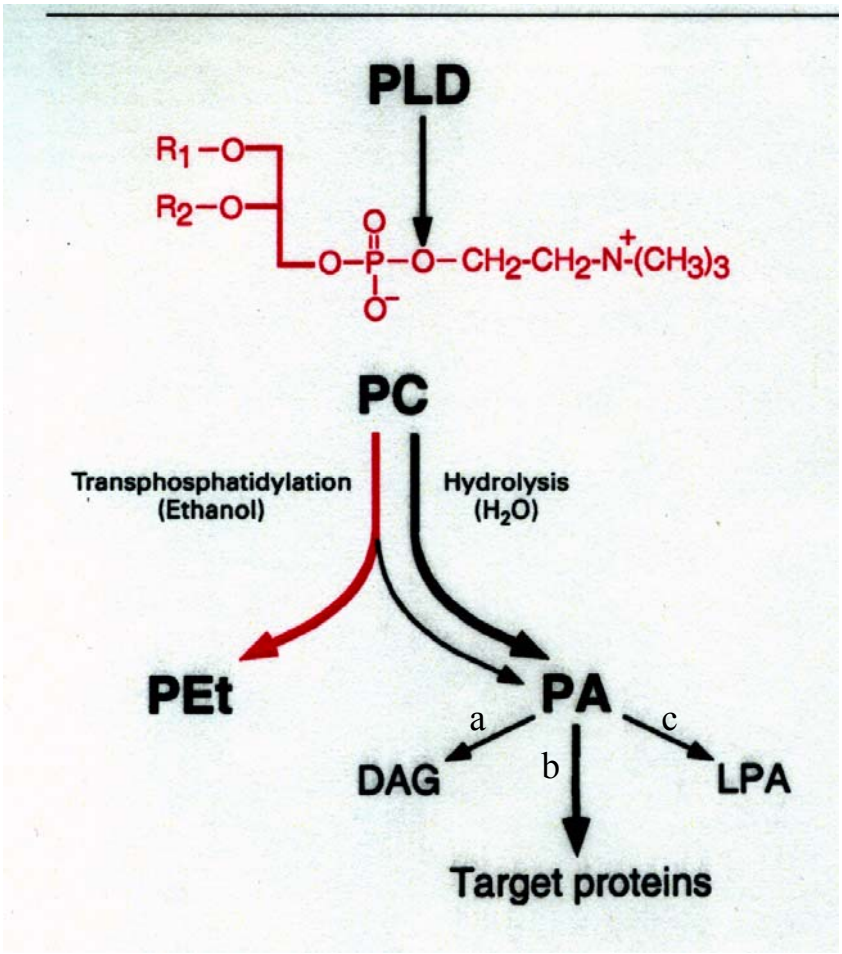


Figure 16. Plasma membrane phosphotidylcholine-directed hydrolytic activity of PLD. PLD hydrolyzes the distal phosphodiester bond linking the phospho group of the choline head group to carbon 3 of the hydrophobic diacylglycerol backbone, generating free choline and phosphatidic acid. A) The PLD cleavage site, illustrating the PLD cleavage site (arrow) on phosphatidylcholine (red). B) A short-lived enzyme-substrate transitional intermediate is formed prior to the liberation of free phosphatidic acid and enzymatic hydrolysis. Primary short-chained alcohols (e.g., ethanol) can compete with water molecules in the PLD hydrolytic reaction to yield a PLD-catalysed transphosphatidylation reaction resulting in formation of a phosphatidic acid ethyl ester or phosphatidylethanol (PEt). PLD-transphosphatidylation (thick red line pointing to the left) occurs at the expense of the hydrolytic reaction (thin black arrow pointing t the right). (a) Phosphatidic acid (PA) is a substrate for phosphatidate phosphohydrolase, which hydrolyses the phosphoric acid group of the third carbon atom on the glycerol backbone yielding diacylglycerol (DAG). Unlike PA and its derivatives (see below), phosphatidylalcohols are metabolically stable, their constitutive levels in cells are normally very low, and they can be readily detected in cells. The PLD-conversion of

(Figure 16 –continued) primary alcohols is therefore a useful measure of PLD activity within intact cells or cell- free systems. In the reverse reaction, DAG is phosphorylated on the third carbon and thus recycled from DAG back to PA. In addition, DAG generated from other membrane-bound hydrolytic activities may also be targeted to increase the local concentration of PA. (b) Aside from its role as a regulator of membrane trafficking, PA is a cofactor for signaling phosphatases and kinases. (c) Once formed, PA may be further metabolized to lysophosphatidic acid (LPA) by other membrane associated enzymes (e.g., PLA_2), wherein the carbon chain extending from carbon two of the glycerol backbone is hydroysed yielding a single chain phospholipid. This figure is from reference (Liscovitch et al., 2000).

(Brown et al., 1993;Cockcroft et al., 1994). Similar studies conducted with Rho family members elucidated the ability of Rho A to directly activate PLD (Bowman et al., 1993;Bowman et al., 1995). Although lacking a pleckstrin homology domain (Sung et al., 1999b), purified PLD, when reconstituted *in vitro* with various phosphoinostide species becomes catalytically active (Hodgkin et al., 2000;Hoer et al., 2000;Sciorra et al., 2002;Zheng et al., 2002;Stahelin et al., 2004;Lee et al., 2005). Additional studies have shown that PLD copurifies with a variety of cellular compartments, including the cytosol (Vinggaard et al., 1997;Exton, 1997), plasma membrane (Provost et al., 1996;Vinggaard et al., 1997), Golgi Apparatus (Ktistakis et al., 1995;Ktistakis et al., 1996), and nucleus (Balboa and Insel, 1995).

One key regulatory aspect of PLD signaling in human neutrophils is its activation downstream of plasma membrane-associated PKC in, for example, chemoattractant receptor-mediated signal transduction cascades (Billah et al., 1989;Kessels et al., 1991;Gelas et al., 1992;Mullmann et al., 1993;Exton, 1994;Olson and Lambeth, 1996;Rais et al., 1998;Wang et al., 2002b;Wang et al., 2003). The PLD pathway is thus strategically situated to regulate the neutrophil cytotoxic potential and, moreover, determine the extent to which microbicidal processes are engaged in response to cellular

stimulation with various types of chemoattractants (Verghese et al., 1987;Truett, III et al., 1988;Kanaho et al., 1991). Significantly, PKC is a central regulator of neutrophil microbicidal function (Berkow et al., 1987;Harvath et al., 1987;Salzer et al., 1987;Gaudry et al., 1988;Smith et al., 1988a;Smith et al., 1988b;Twomey et al., 1990;Twomey et al., 1991;Tanimura et al., 1992;Kessels et al., 1993;Cabanis et al., 1996), and the activity of this enzyme is contingent on the availability of various co-factors, including diacylglycerol (DAG) (Iwata et al., 1990), a product of both PLC-phosphoinositide and PLD-phosphocholine metabolic pathways (Figures 14 and 16). Indeed, the variable capacities of peptide- and lipid-based chemoattractants to co-induce heptahelical receptor-activated microbicidal function during cellular chemotaxis strongly correlates with the ability of each type of chemoattractant to elicit PLD activation after receptor engagement (Verghese et al., 1987;Truett, III et al., 1988;Kanaho et al., 1991). Importantly, PLD activation is bimodal in chemoattractant-stimulated neutrophils, with temporally distinct, concomitant phases of enzyme activity. The later phase of PLD activation determines the strength and duration of downstream DAG-dependent PKC activity that bridges the principle effector response of directed motility with PKC-dependent signaling processes initiating superoxide generation and granule exocytosis (Garland, 1992;Yasui et al., 1994). Accordingly, this latter phase of PLD activity is only transiently induced in response to lipid chemoattractants, while stimulation with peptide chemoattractants results in sustained PLD activity. Accordingly, the differential regulation of PLD activity in leukocytes could contribute to the observed graded-state of neutrophil activation depicted in Figure 12.

Because certain chemoattractants, like fMLF, selectively induce the sustained activation of PLD required to further engage microbicidal signaling pathways (Parkos et al., 1985;Petrequin et al., 1986), they may be viewed as molecules that have evolved to engage a select subset of effectors. The organization of such effectors within leukocyte plasma membranes is a primary focus of current research, which suggests that pathway-specific components may exist in supramolecular assemblies that spatially and temporally modulate signal transfer. This organization, in turn, could regulate the accessiblity of receptors to downstream signaling and effector proteins (Karnell et al., 2005;Zhang et al., 2005;Hermida et al., 2006) (Harris et al., 2001;Ito et al., 2004) and thus affect the kinetics of signal transmission (Sproul et al., 2000;Bromley et al., 2001;Darlington et al., 2002;Meiri, 2005;Fabre et al., 2005;Round et al., 2005). A major mechanism underlying these processes includes the selective segregation of receptors and/or signaling components into structurally distinct membrane-associated compartments (Sako and Kusumi, 1994;Simson et al., 1998;Ritchie and Kusumi, 2004;Harder and Engelhardt, 2004;Meiri, 2004;Golub et al., 2004;Kusumi et al., 2005). Such compartments are multifunctional, designed in part to insure that non-activated cells maintain their quiescent phenotype and, conversely, to enhance the efficiency of essential microbicidal and related cellular processes by the targeted delivery of individual and/or pre-assembled pathway components to sites of receptor engagement. Various spatially directed functional processes, including leukocyte phagocytosis and chemotaxis, depend on the regional amplification of receptor signals that is facilitated by the initial, selective sequestration of signaling components within plasma membrane. It is this concept

that provides a primary basis for modeling the dynamic and complex biochemical and morphological processes that govern leukocyte function.

The Plasma Membrane as a Chemosensory Apparatus. Notably, some investigators have predicted that resting, eukaryotic plasma membranes are "equipotent" in their chemosensory behavior and that the localized activation of signaling pathways confers this extraordinary capability (Jugloff and Jongstra-Bilen, 1997;Parent et al., 1998;Weiner et al., 1999;Parent and Devreotes, 1999;Servant et al., 1999;Servant et al., 2000). The equipotency hypothesis predicts that the ability of membrane receptors to respond to chemotactic stimuli depends, not on the specialized microdomain distribution of receptors nor their functional segregation in resting plasma membranes, but on the uniform distribution of functionally equivalent receptors. Processes intrinsic to the membrane itself will then propagate and amplify signals from receptors facing the chemoattractant source while inhibiting chemoattractant signaling in all other membrane regions. This viewpoint emphasizes that the localized activation of signaling pathways facilitates direction finding and thus initially directs neutrophil polarization through a process of signal amplification during cellular exposure chemoattractant gradients. Intracellular signaling gradients are significantly steeper than the chemoattractant gradient across the length of the cell, which may be as small as 1-2% (Zigmond, 1974). Signaling gradients are generated before acquisition of morphological polarity and are therefore proposed to underlie polarity development (Rickert et al., 2000;Servant et al., 2000;Wang et al., 2002a;Schneider and Haugh, 2005;Pankov et al., 2005). This model is supported by findings that (i) molecules

responsible for generating signaling gradients also serve as primary effectors of cytoskeletal reorganization and pseudopod formation; and that (ii) inhibition of the signal amplification loop also negates polarity development. Because signal amplification occurs downstream of receptor activation, signaling gradients in chemoattractant-activated neutrophils are largely assumed to occur independently of enhanced receptor-signaling strength conferred by active redistribution of surface membrane receptors to the leading edge of chemoattractant-stimulated cells (Servant et al., 1999). Thus, in the case of the FPR, agonist-ligated receptors remain uniformly distributed on the micrometer scale of the cell surface during fMLF-mediated chemotaxis (Servant et al., 2000).

PI-3K and Rho GTPase Activity in Chemosensory Behavior. The proposed limiting factor of chemotattractant signal amplification is phosphoinositide-3 kinase (PI-3K) (Firtel and Chung, 2000). PI-3K is the central mediator of a positive feedback loop that coordinates Rho GTPase activation with PIP_3 formation in chemoattractant-stimulated leukocytes (Wang et al., 2002a;Niggli, 2003;Weiss-Haljiti et al., 2004;Wu, 2005;Procko and McColl, 2005). PI-3K activity forms a biochemical basis for the establishment of polarity in chemoattractant-stimulated leukocytes through the compartmentalized, selective activation of PH domain-containing proteins that regulate Rho GTPases. The respective activities of Rho GTPases, Cdc42 and Rac, in turn act to localize/restrict and amplify the PI-3K-generated signaling activity gradient. For example, C5a-activated neutrophils from mice deficient in PIXα, a Cdc42-specific guanine nucleotide exchange factor, can adopt a

polarized morphology but fail to navigate up a concentration gradient of chemoattractant (Li et al., 2003). The normal and deficient levels of activated Rac and Cdc42, respectively, in C5a-stimulated, PIXα-deficient neutrophils suggests that Rac GTPase mediates F-actin polarization and pseudopod formation. However, the errant polarization behavior of PIXα-deficient neutrophils (i.e., in an orientation away from the C5a chemoattractant source) indicated an essential role for Cdc42 coordinating the directionality of cell polarization with chemoattractant receptor activation and signaling amplification processes. Other studies used transient transfection of Rac GTPase with or without dominant interfering proteins to confirm the role of Rac as the primary effector GTPase in F-actin accumulation, pseudopod development and cellular polarity (Srinivasan et al., 2003;Park et al., 2004;Dong et al., 2005). A similar experimental approach was used to verify that Cdc42 activity regulated the number, stability and the directionality of pseudopods formed in chemoattractant-stimulated leukocytes (Allen et al., 1998;Srinivasan et al., 2003). Collectively, these studies also suggest the importance of secretory vesicle membrane-plasma membrane fusion events in neutrophil chemotaxis. This connection arises in view of the direct association of PI3-K with Rac GTPase (Tolias et al., 1995). Significantly, analogous protein complexes have been shown to localize preferentially and rapidly to intact, intracellular secretory vesicles during the course of their exocytosis in activated neutrophils (Kobayashi et al., 1998a).

Biochemistry of Actin

Regulation in Chemotaxing Cells. The initial, rapid disassembly of existing actin networks is considered prerequisite for cell migration (Mouritsen and Bloom, 1993).

The gelsolin family of actin binding proteins is believed to be one of the central effectors of this process (Stossel et al., 1999). This inference is grounded in two unique properties of this family. These include i) effector potential, which encompasses blocking, severing, and nucleating activities (Fujita et al., 1995); and ii) capacity for regulation by phosphoinositides, calcium, and hydrogen ions (Janmey and Stossel, 1989). In contrast, the effector function of another class of actin severing protein, the cofilin/destrin family, is regulated through serine-threonine phosphorylation (Nagaoka et al., 1996). The potential significance of this latter family is indicated in studies showing that cell migration is maintained in gelsolin-knockout mice (Witke et al., 1995). Actin severing proteins, intracellular calcium and membrane phosphoinositides work in conjunction with actin-crosslinking, capping and nucleating proteins to regulate the kinetics of cytoskeletal rearrangement and F-actin accumulation within developing pseudopodia conferring cell polarization and directed migration.

One confounding aspect of current paradigms depicting a molecular basis for chemoattractant-induced actin cytoskeletal remodeling involves the timing between initial stimulation with chemoattractant and the kinetics of cellular responsiveness in terms of actin rearrangement. In such models, cyclic elevations in intracellular calcium during chemotaxis are thought to promote the periodic disassembly of the pseudopodial F-actin network required for continued migration through the targeted inhibition of actin crosslinking proteins and the activation of F-actin severing proteins. A diversity of calcium-regulated activities is thought to be required for actin remodeling, including the severing activity of gelsolin (Janmey and Stossel, 1989) and the actin-binding activity of

all major classes of filament cross-linkers found in neutrophils, including filamin (Sobue et al., 1982;Watts and Howard, 1994), α-actinnin (Reddy et al., 1983;Watts and Howard, 1994;Jones and Brown, 1996), and fimbrin (Bretscher and Weber, 1980). In order for cell polarization to occur, the F-actin networks existing in nonactivated cells must be dissassembled and reorganized, so elevations in intracellular calcium would thus be expected to precede pseudopod development. Experimentally, however, pseudopod formation precedes detectable increases in intracellular calcium by as much as three seconds following fMLF exposure (Demaurex et al., 1994), occurring within two-to-five seconds after agonist addition (Gerisch and Keller, 1981;Davis et al., 1982). Similarly, activation of the cofilin/destrin family of actin severing proteins likely occurs downstream of PKC (Nagaoka et al., 1996), the enzyme responsible for capacitive increases in intracellular calcium, and is also inconsistent with a role for intracellular calcium-flux in pseudopod development. Hence, the mechanism of cortical actin disassembly in the absence of calcium remains elusive.

Interestingly, there is good evidence that pseudopod formation may be attributable to a closed, positive feedback loop involving the Rac GTPase-mediated generation of phosphatidylinositol 4,5-bisphosphate at the leading edge of migrating cells (Benard et al., 1999;Chung et al., 2000). In this popular and widely accepted model system, there is strong evidence that plasma membrane phosphoinositides chiefly regulate the assembly and subsequent stabilization of filament networks (Cunningham et al., 2001). These findings would support the contention that localized supplementation of membrane, through targeted exocytosis, rather than the disentanglement and subsequent

expansion of existing, membrane-skeletal-bound plasma membrane, is central to the extension of pseudopod at the leading edge of migrating cells. In line with this proposal, the clearance of membrane skeletal components from pre-fusion sites in plasma membranes would presumably require relatively minor, spatially confined alterations in the existing skeletal network (Almers, 1990). Such alterations could quite possibly be accomplished by phosphoinositide-mediated uncapping of actin molecules for subsequent actin remodeling and membrane extension, independently of intracellular calcium levels and/or PKC-dependent serine/threonine phosphorylation of actin severing proteins. Such activities, under defined conditions, could thus result in the dynamic disassembly of existing actin filaments in the absence of calcium (Yu et al., 1990;Haus et al., 1991;Heiss and Cooper, 1991;DiNubile et al., 1995;DiNubile and Huang, 1997). Endosomal fusion would then be afforded by the D_4-phosphoinoside-dependent recruitment fusion machinery (Cockcroft, 1996;Corvera et al., 1999;Cockcroft and De Matteis, 2001). It may therefore be predicted that, in fMLF-directed neutrophil chemotaxis, the observed enrichment of D_4 phosphoinositides within the FPR activation domain (Fig 15), PML, may be central to the mechanism of endosomal-plasma membrane fusion events by:

i) Initially introducing dynamic instability into existing skeletal networks through the sequestration of capping proteins.

ii) Directing endosomal fusion events.

iii) Facilitating actin polymerization in post-fusion membranes, possibly in a Rac-dependent manner (Tolias et al., 1995;Benard et al., 1999;Belisle and Abo, 2000).

A Conceptual Basis for Understanding Leukocyte Chemosensory Behavior. One critical point in the equipotency hypothesis (see above) that remains to be addressed concerns how the simultaneous down-regulation of receptors in peripheral (i.e., non-leading edge) membranes is accomplished. Indeed, the equipotency hypothesis as well as the counter viewpoints supporting receptor redistribution as a basis for signal amplification (Firtel and Chung, 2000;Chung et al., 2001) both imply that spatially restricted signal amplification forms the biochemical and morphological bases of polarization in neutrophils. In support of the latter concept, Mackay et al. (McKay et al., 1991) and others (Zigmond et al., 1981;Walter and Marasco, 1984) have indicated that FPR-redistribution to the leading edge of migration is paramount in directed migration.

Importantly, much of the controversy over the molecular basis of cellular polarity in neutrophils may be resolved by considering basic aspects of neutrophil physiology and the dynamic physical and biochemical alterations that occur in chemoattractant-activated cells. Such observations suggest a basis for neutrophil polarity that is attributable to the localized activation of signaling pathways, as stated by the equipotency hypothesis, in conjunction with the spatial compartmentalization of signal generation. A physiologic basis for the confinement of signal amplification to the leading edge membrane is suggested by the previously discussed temporal characteristics of secretory vesicle mobilization in activated neutrophils. T cells contain a similar compartment that is upregulated upon initial stimulation (Pradhan and Morrow, 2002). While the kinetics of secretory vesicle exocytosis in neutrophils (which requires microtubules) are consistent with their possible involvement in pseudopod formation and/or extension, the capacity of

secretory vesicles to specifically target and fuse within a morphologically defined membrane region remains to be shown. One possible mechanism that could underlie the proposed membrane targeting behavior of this organelle in neutrophils (and analogous organelles in other leukocytes (Pradhan and Morrow, 2002)) involves the regulation of directional targeting of exocytosis in relation to the relative proportion of agonist-occupied surface receptors per area of membrane. This contention is indirectly supported by findings from several studies:

i) The rapid re-orientation of the centrosome-associated microtubule cytoskeleton toward leading edge of chemoattractant-stimulated neutrophils (Malech et al., 1977).

ii) The microtubule-dependent targeting of secretory vesicles to developing lamellae in some mammalian cells (Bergmann et al., 1983;Hopkins et al., 1994).

iii) Long-standing observations regarding the preeminent role of microtubules in the processes of orientation and direction finding in neutrophils (Zigmond, 1977;Oliver, 1978).

iv) Observations of secretory vesicle membranes as the primary source of NADPH oxidase assembly and superoxide generation, indicating a primary role for this organelle in neutrophil effector function (Kobayashi et al., 1998a;Robinson et al., 1999).

v) Preferential targeting of internal membranes associated with NADPH oxidase activity to regions of surface membrane involved in the phagocytic uptake of infectious particles (N'Diaye et al., 1998;Perskvist et al., 2002;Cougoule et al., 2002;Vieira et al., 2003). Significantly, the targeted assembly and functional activation of the NADPH oxidase complex on activated neutrophil plasma membranes occurs selectively within the PMH domain (Quinn et al., 1989).

In addition, because FPR is equally distributed between resting plasma membranes and secretory organelles of human neutrophils (Sengelov et al., 1994a), initial endosomal-plasma membrane fusion events could lead to the appearance of receptor redistribution (and accumulation) at the leading edge of directed migration, thus accounting for the mixed observations of chemoattractant receptor clustering in pseudopodial membranes. However, targeted exocytosis of FPR-enriched granules (e.g., secretory vesicles) could account for a transient accumulation, as receptors may redistribute over time in post-fusion membranes and achieve uniformity. Alternatively, others have suggested that chemoattractant receptors may dynamically redistribute in primed plasma membranes at various stages of neutrophil chemotaxis (Sullivan et al., 1984;Walter and Marasco, 1984;Servant et al., 1999). This would, furthermore, be consistent with observations that agonist-activated FPR in neutrophil plasma membranes lose their mechanical restriction to PML compartments with time, arriving at cytoskeletally anchored sites in PMH. These observations are supported by evidence

demonstrating that PML is depleted cytoskeleton-protein content (Jesaitis et al., 1988;Jesaitis and Klotz, 1993).

FPR-Chemotaxis in Human Neutrophils.

Many questions pertaining to the organization of FPR in relation to downstream effectors and bioactive lipid classes persist. In particular, there is a considerable gap in knowledge relating to the nature of signal transfer and subsequent generation of signaling gradients is organized and/or regulated by key structural intermediates identified in neutrophils (e.g., membrane rafts and various types of protein scaffolds) that play an important role in linking receptor signaling processes to specific functional outcomes. This section will discuss the roles of (i) membrane lipid and cytoskeletal microdomains and (ii) the potential contribution of secretory vesicle exocytosis in neutrophil chemotaxis, with emphasis on relative significance in FPR-function where possible. This section begins with a brief description of FPR signaling properties in primary and heterologous systems.

Biochemistry of FPR-
Chemotaxis in Heterologous Systems. Functional studies in heterologous expression systems examining fMLF-directed chemotaxis have firmly established the essential role of inducible ‘activation’ domains in FPR compartmentalization and the spatially restricted amplification of FPR-generated signaling cascades (Servant et al., 2000). In key experiments, GFP fusion technology and quantitative fluorescence microscopy were coupled to enable real-time observation of select downstream signaling

intermediates within intact, fMLF-stimulated cell lines. Thus, transient expression of a GFP-PKB fusion protein facilitated characterization of activation-induced signaling gradients that in turn revealed a molecular basis of FPR-transduced polarization and directed migration. Central aspects of this amplification cascade include localized modifications in plasma membrane lipid and skeletal composition that occur within the developing leading edge membranes.

Mechanisms of FPR-Chemotaxis in Human Neutrophils. In neutrophils, the formation of chemoattractant-induced "activation" domains involve:

i) Receptor-G protein coupling within G protein enriched domains (Jesaitis et al., 1988;Keil et al., 2003).

ii) Local alterations in the metabolism of select phospholipid classes, including phosphatidylinositols (PI) (Servant et al., 2000), which exist as discreet clusters within the inner leaflet of leukocyte plasma membranes (Parmryd et al., 2003).

iii) Focal activation of enzymatic effector systems, including inositol kinases/phosphatases (Petrie et al., 2000;Seveau et al., 2001a;Galandrini et al., 2002;Wang et al., 2002a) and low molecular weight (LMW) GTPases (Gardiner et al., 2002;Srinivasan et al., 2003), that preferentially localize within membrane pockets enriched in saturated lipids.

iv) Focal induction of actin polymerization, a process mediated by positive feedback between the LMW GTPase, Rac 2, and select PI lipid species (Xu et al., 2003;Zadeh and Keller, 2003).

This structural heterogeneity of plasma membranes, it would seem, contributes to the origin and development of FPR-directed cellular polarization and subsequent functional differentiation not only by organizing signaling cascades but also by providing a biochemical basis, or driving force, for the focal amplification of signals. This view of FPR activation in neutrophils emphasizes the functional importance of plasma membrane domains in receptor function. Indeed, preliminary experiments examining of relative lipid content of PML/PMH domains indicate differential enrichment of phosphoinositide phospholipids and cholesterol (Fig 15). In addition, enrichment of morphogenic lipid intermediates, such as phosphatidic acid, within both domains supports the possibility that the regulation of FPR chemotaxis occurs within these compartments.

Role of Plasma Membrane Lipid Organization in FPR-Directed Neutrophil Polarization and Chemotaxis. Several recent studies have demonstrated the importance of plasma membrane lipid organization in neutrophil polarization, chemoattractant receptor signaling, and directed migration, thus implicating a primary role for membrane rafts in FPR-mediated signal transfer and related downstream effector activties. The saturated acyl chains of the phospholipid species contributing to raft structure are proposed to confer a phase shift in local bilayer organization from a fluid to a liquid-ordered (l_0) physical state. This phase shift results from the tight hydrophobic association of adjacent raft-lipids with cholesterol and forms

the basis of the relative insolubility of membrane rafts following solubilization of cells or cellular membranes in cold (4°C) nonionic detergents. Just as *in vitro* isolation of detergent-insoluble membranes by flotation on sucrose density gradients is widely accepted as proof of their *in vivo* existence within intact cellular membranes, similarly indirect methods are also employed to provide evidence of raft-involvement in cellular signaling processes. These latter methods are based primarily on evidence demonstrating the cholesterol-dependence of membrane rafts in membrane model systems and involve cellular treatments with either specific chelating agents that deplete plasma membranes of cholesterol content or that metabolically inhibit cholesterol synthesis (Bastiaanse et al., 1995;Pediconi et al., 2004;Yu et al., 2006;Somogyi et al., 2006). These experimental techniques have been employed in neutrophil studies to demonstrate the accumulation detergent-resistant plasma membrane domains that dynamically form in response to FPR activation. Once formed, membrane rafts coalesce and form a cap that extends over the central portion of the cell body and uropod of activated neutrophils during the course of cell polarization (Seveau et al., 2001a). Cellular incorporation of raft-preferring fluorescently labeled lipid analogs prior to fMLF-stimulation support detergent studies in suggesting the differential redistribution of order-preferring phospholipids in polarized neutrophils towards the rear of the cell, with polyunsaturated phospholipids accumulating within the leading edge membrane (Seveau et al., 2001a;Pierini et al., 2003).

Other studies demonstrate the essential nature of plasma membrane phospholipid heterogeneity in key functional processes of human neutrophils. Following incubation with cholesterol depleting agents or metabolic inhibitors, neutrophil populations fail to

polarize and chemotax in response to isometric or point gradients of fMLF (Pierini et al., 2003), indicating that fundamental properties of neutrophil function are regulated by local changes in phospholipid composition. In addition, certain intracellular signaling pathways induced in response to fMLF-stimulation appear to rely on the structural integrity of membrane rafts for signal transfer. For example, neutrophils depleted of plasma membrane cholesterol prior to fMLF-stimulation are inhibited in FPR-transduced capacitive increases in intracellular calcium required for granule secretion (Barabe et al., 2002b;Pierini et al., 2003) and also fail to activate the extracellular regulated kinase (ERK) branch of the mitogen-activated protein kinase (MAPK) family. This branch of the MAPK pathway is an important contributor of neutrophil chemotaxis (Hii et al., 1999), activation of the oxidative burst, and the induction of phagocytosis (Downey et al., 1998). Lipid rafts also regulate activation of Rho GTPases Cdc42 (Fessler et al., 2004) and Rac (Pierini et al., 2003) in stimulated human neutrophils. Cdc42 is required for both the proper orientation of polarized neutrophils toward the chemoattractant source and stabilization of pseudopodial membranes (Oliferenko et al., 1999), whereas Rac is necessary for assymmetric F-actin remodeling (Pierini et al., 2003;Srinivasan et al., 2003) required for the induction of polarization in fMLF-activated neutrophils (Watts et al., 1991;Coates et al., 1992). The apparent functional dependence of fMLF-activated neutrophils on membrane rafts coupled with the redistribution of raft-phospholipids in polarized plasma membranes suggests that microdomain assembly is coupled with cellular structures that enable their active reorganization in polarized cells.

In several instances, cooperative interactions between membrane rafts and structural elements of the membrane skeleton were shown essential to the induction of cellular activity following FPR-stimulation. Transmembrane receptor proteins are the foremost candidates in the proposed associations of membrane rafts with structural components of the membrane skeleton in human neutrophils (Pierini and Maxfield, 2001). The leukosialin receptor, CD43, and hyaluronin receptor, CD44, are heavily glycosylated proteins implicated in coordinated raft-skeleton rearrangements (Oliferenko et al., 1999;Seveau et al., 2001a), though their specific roles in neutrophil function and signal transduction are largely undefined. Both transmembrane proteins interact concomitantly with membrane rafts and membrane skeletal components seconds after fMLF-activation (Seveau et al., 2001a). Neutrophil polarization is thus coupled to the reorganization of plasma membrane-bound CD43 and CD44 from a random distribution in nonactivated neutrophils to one that is polarized toward the cell body and uropod. CD43 and CD44 rearrangements in chemoattractant stimulated plasma membranes are inhibited in neutrophils pre-incubated with the myosin light chain kinase inhibitor, ML7, which also inhibits the morphological polarization of stimulated cells. On the basis of this latter observation, it was proposed that CD43/CD44 redistribution provided a basis for polarity development in human neutrophils (Seveau et al., 2001b). This notion, however, was dispelled in later studies showing the rearrangement of CD43 to be consequence rather than a prerequisite for polarity development (Dehghani et al., 2003). In addition, CD43 associates with the membrane skeleton by the ezrin-radixin-moesin (ERM) family of molecules (Yonemura et al., 1993;Serrador et al., 1998;Allenspach et al., 2001), which

link the receptor cytosolic domain to cytoskeletal F-actin (Sechi and Wehland, 2000;Bretscher et al., 2002). The ERM family members expressed in human neutrophils include ezrin and moesin, which are very often under the regulatory control of membrane phosphoinositides, principally PIP_2 (Louvet-Vallee, 2000;Bretscher et al., 2000).

PIP_2 and possibly related phosphoinositides are preferentially enriched within the activation domain of the FPR, PML (Fig 15). It is thus possible that the subcellular localization pattern of CD43 and ezrin in activated neutrophil plasma membranes may offer additional clues to the physical, biochemical and functional nature of this compartment. The value of such data would depend on validation of the colocalization of CD43 and ezrin with PML marker proteins. Short of this, colocalization cannot be inferred. However, the detection of specialized plasma membrane functional compartments following shear-induced fragmentation of neutrophil membranes in density separations may also be indicated (see below).

The work described in Chapter 3 uses established functional and/or structural relationships between several proteins of interest, including CD43 and ezrin, to support the physiological relevance of density-isolated, functionally specialized plasma membrane compartments within nonactivated and/or activated (primed) neutrophils. This work demonstrates the codistribution of CD43 and ezrin at a density of ~ 34% sucrose in activated plasma membranes, a ~ 4% shift in sucrose density from the peak distribution of the plasma membrane marker protein, alkaline phosphatase, at ~ 29% sucrose. Other experiments not included in Chapter 3 additionally indicate a peak subcellular distribution of the PML domain at ~ 34% sucrose. This latter data is from experiments

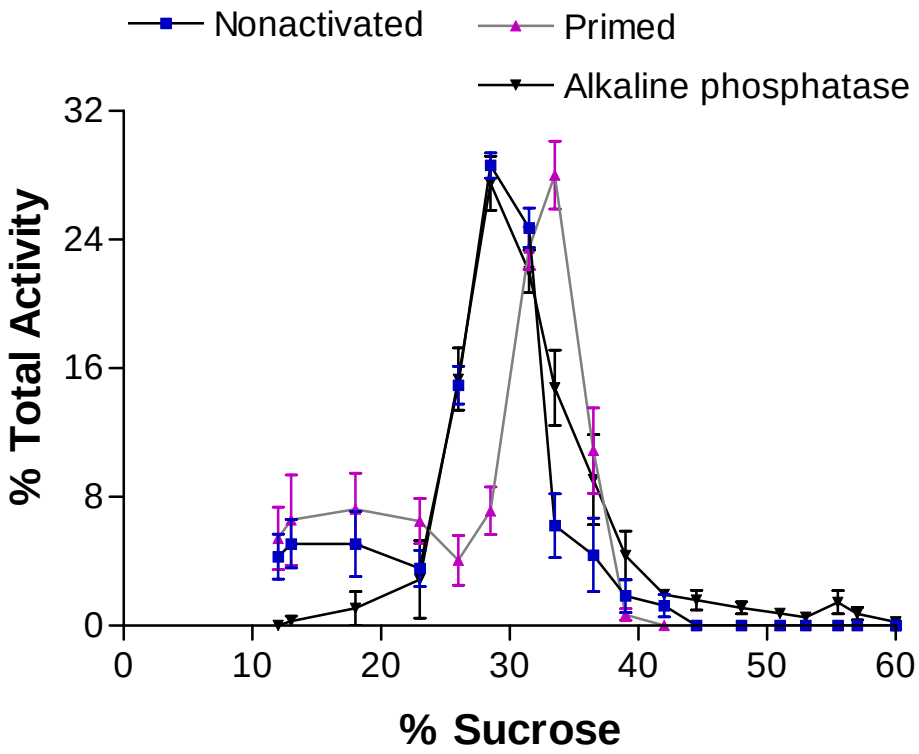


Figure 17. The differential distribution of the FPR-specific heterotrimeric G protein subunit, $G\alpha_{i\text{-}2}$, in resting (nonactivated; blue, squares) versus primed (purple, triangles) human neutrophils. Human neutrophils were isolated one-unit blood preps using either a dextran-based (blue, squares) or a gelatin-based (purple, triangles) approach. The dextran-based methodology was performed under aseptic conditions using sterile, endotoxin-free reagents and plasticware and allowed isolation of neutrophil populations in a non-activated cellular state. Analysis of this population in the above figure demonstrates the absence of the PML microdomain in non-activated neutrophil plasma membranes, and thus consistent with the dedicated function of this domain in terms of FPR activation. In particular, the peak concentration of $G\alpha_{i\text{-}2}$ in this neutrophil population precisely coincides with that of alkaline phosphatase (black, inverted triangles), indicating a random, e.g., nonspecific/noncompartmentalized, distribution in plasma membranes. Failure to detect PML in nonactivated plasma membranes further indicates the latent, inactive nature of resting plasma membranes through deliberate sequestration of activated (i.e., perturbable or signaling competent) membranes in the form of secretory vesicles (see text). The second technique employed for blood-neutrophil isolation is the gelatin-based approach which affords the isolation of primed (activated) neutrophil populations. The presence of the PML domain in this population is clearly depicted by the relative shift of $G\alpha_{i\text{-}2}$ into higher sucrose densities, with a peak distribution at ~34%. The alkaline phosphatase activity shown represents the averaged activity from both nonactivated and primed neutrophil populations examined. These data

(Figure 17 –continued) are averaged from six separate experiments using a total of three nonactivated and three activated populations.

similar to those conducted in Chapter 3 and is presented in Figure 17, which shows the subcellular distribution of the PML marker protein, $G\alpha_{i\text{-}2}$, in density gradients prepared from nonactivated and primed human neutrophils.

The CD43 and ezrin density distributions presented in Chapter 3 are informative from the standpoint of ezrin function. Ezrin preferentially associates with and anchors certain of membrane receptor families to the cell cortex, including mucosialoglycoproteins, CD43 and CD44. The well-described functional association of these molecules in leukocytes coupled with their codistribution at ~ 29% sucrose in gradients prepared from nonactivated neutrophils and their coordinated shift in subcellular localization to ~34% sucrose observed in primed plasma membranes could suggest the co-compartmentalization of these molecules in both resting and primed neutrophils (Chapter 3). In view of these findings, the data shown in Figure 17 demonstrating that $G\alpha_{i\text{-}2}$ undergoes an equivalent shift in percent sucrose localization in primed plasma membranes suggests several interesting working hypotheses on which to base future investigations. Several examples are listed below:

i) *Colocalization CD43 and ezrin with $G\alpha_{i\text{-}2}$ in primed neutrophils.* Validation of the colocalization of these proteins is necessary to justify the additional experiments listed below (ii-iv). One basic approach would entail the immunoprecipitation of CD43, ezrin or $G\alpha_{i\text{-}2}$ from gradient fractions that are coenriched for these proteins, followed by confirmation of the other

two proteins by immunoblot analysis. One limitation of coimmunoprecipiation is that this approach relies on a stable physical interaction between components, which need not be true for the compartmentalization of functionally unrelated proteins within the same microdomain. To address such possibilities, the colocalization of molecules within a given microdomain could be carried out by methods similar to those described in Chapter 4, where membrane subfractions were immunoisolated from homogenates using specific monoclonal antibodies. In particular, protein complexes forming as a result of interactions between individual signaling components and the ordered bilayer matrix of phase-shifted (gel-like) sphingo/glycolipids, the underlying cytoskeletal structure, or both structures could be examined. However, if negative results are obtained in coimmunoprecipitation studies, an additional, more direct method for showing protein-colocalization in microdomains using direct or indirect immunofluorescence microscopy could then be applied.

ii) *The redistribution of FPR functional domains, PML/PMH, in polarized neutrophil plasma membranes.* Importantly, the primed populations used in the experiments described in Chapter 3 and Figure 17 are not polarized. Nonetheless, FPR functional domains (PML, PMH) are selectively assembled in this population relative to nonactivated neutrophils. The presence of these functional domains in nonpolarized membranes in

addition to their distinct differences in molecular composition can offer an experimental basis on which to track the mobility these structures during neutrophil polarization. The data described above relating to CD43 mobilization in neutrophil membranes could suggest several possible outcomes from the experimental tracking of a PML or PMH marker protein. First, it is known that the myosin light chain kinase inhibitor, ML-7, inhibits both neutrophil polarization and CD43 redistribution in response to fMLF-stimulation. This would suggests that neutrophil polarization and CD43 redistribution are linked at a molecular level. Further, CD43 localization in polarized neutrophils occurs within regions of cortical F-actin enrichment. CD43 also cosediments with the PML marker, $G\alpha_{i\text{-}2}$ (see above).

Assuming that CD43 does in fact colocalize with $G\alpha_{i\text{-}2}$, in activated, nonpolarized plasma membranes, several functional implications become evident. The first would be that PML and/or PMH redistributes during the course of polarization to distinct regions of the plasma membrane (Fig 18). A second possibility is that CD43 (in this case, a hypothetical marker of PML) moves out of PML during the course of polarization and localizes to PMH. This is suggested by the redistribution of CD43 to cortical actin-enriched uropods in fMLF-stimulated neutrophils (actin-enrichment being one of the defining characteristics of the PMH domain). A third outcome could be that PML undergoes major compositional changes during the

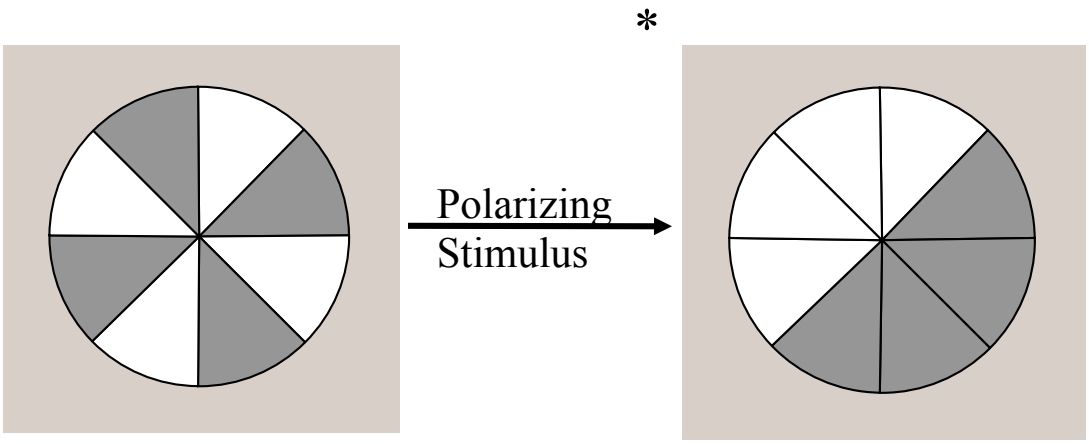


Figure 18. Predicted distribution patterns of PML (white) and PMH (gray) in primed but nonpolarized (left) and polarized (right) neutrophils. During polarization neutrophils actively redistribute membrane proteins to either the developing leading or trailing edge membranes during initial polarization leading to the formation of membrane compartments that are specific to defined morphological regions of cells. The spatial organization of functional domains of the FPR, including the PML activation domain and the PMH desensitization domain, in activated but nonpolarized (left) and polarized (right) plasma membranes is not known. However, the spatial distribution of these functional domains in polarized plasma membranes (right) may be predicted on the basis of their functional properties. As segregation of agonist-bound FPR into the PML domain enables the efficient engagement of receptor-specific pathway components required for the rapid transfer of signals, this domain may localize to the leading edge of migrating cells, where sustained signal amplification occurs. Conversely, the sequestration of activated FPR into the PMH domain, which is devoid of signaling proteins and enriched with cortical cytoskeletal proteins that complex with and deactivate receptors for subsequent removal from the cell surface. This specialized role of PMH in FPR regulation may therefore indicate localization to the trailing edge of migrating cells. The organization of PML and PMH in activated but nonpolarized cells (left) is also presented and is proposed to assume a random distribution. The asterisk indicates a point source of chemoattractant, with the leading edge membrane of polarized cells positioned toward this stimulus.

process of polarization. Because PML is an activation domain of FPR, neutrophil stimulation with fMLF should promote in PML localization within pseudopods, which is the only region of the plasma membrane housing activated agonist-coupled receptor. Such a localization would be consistent with the transition of CD43 from PML to PMH and the subsequent clustering CD43 (and PMH) within cortical actin-enriched uropods. One final possibility is suggested by the observation that CD43 redistribution is mechanistically linked with induction of the polarization process, which implies that PML (or PMH) may in some way facilitate or contribute to the polarization process.

iii) *Molecular basis for the assembly of FPR functional domains in activated neutrophil plasma membranes.* In view of the absence of PML/PMH in nonactivated neutrophils and their assembly in activated, nonpolarized plasma membranes, it may be possible to explore the molecular requirements of their formation.

iv) *Correlating the role of membrane rafts and PML/PMH function with FPR activation, signal transfer, and the downstream function of effector proteins in polarized neutrophils.* One basic question that remains to be definitively addressed is the role of membrane rafts in FPR function. Several findings suggest a potential relationship between PML/PMH and membrane rafts, including the importance of the cortical domains in FPR regulation, the cooperative associations of cortical proteins with

membrane raft components in leukocytes, the redistribution of membrane rafts in polarized neutrophils, the heterogeneity of membrane rafts in terms of lipid and protein composition (Shogomori and Brown, 2003;Chamberlain, 2004;Lommerse et al., 2004), and the critical importance of rafts in transmembrane receptor signal transduction.

Correlating the Morphological and Functional Polarization with the Regulated Exocytosis of Secretory Vesicles. The observed rapidity of pseudopod formation discussed in the preceding sections may be attributable to misidentification of the cellular state of activation (see Proposed Biochemical Standardization of the Neutrophil Activation State). Evaluation of the secretory vesicle content of purified neutrophils provides a reliable quantitative measure of neutrophil activation state. Importantly, the above-cited studies examining neutrophil chemotaxis distinguish nonactivated cells on an empirical basis, in spite of evidence detailing the marked instability of secretory vesicles within *in vitro* isolated, non-activated neutrophils (Borregaard et al., 1987). Indeed, the recovery of either non-activated or activated neutrophils from whole blood is possible, depending on the method employed for their isolation (Figure 10; Chapters 2-3). Activated neutrophils (i.e., neutrophil populations that become activated during the course of blood-isolation apart from deliberate exposure to agonist) display a significantly greater functional response upon agonist stimulation relative to nonactivated cells (Chapter 3), yet may be classified as 'nonactivated' by empirical methods based on the absence of agonist exposure (Chapter 2). Because the time required for pseudopod development and cell-polarization is likely to vary in

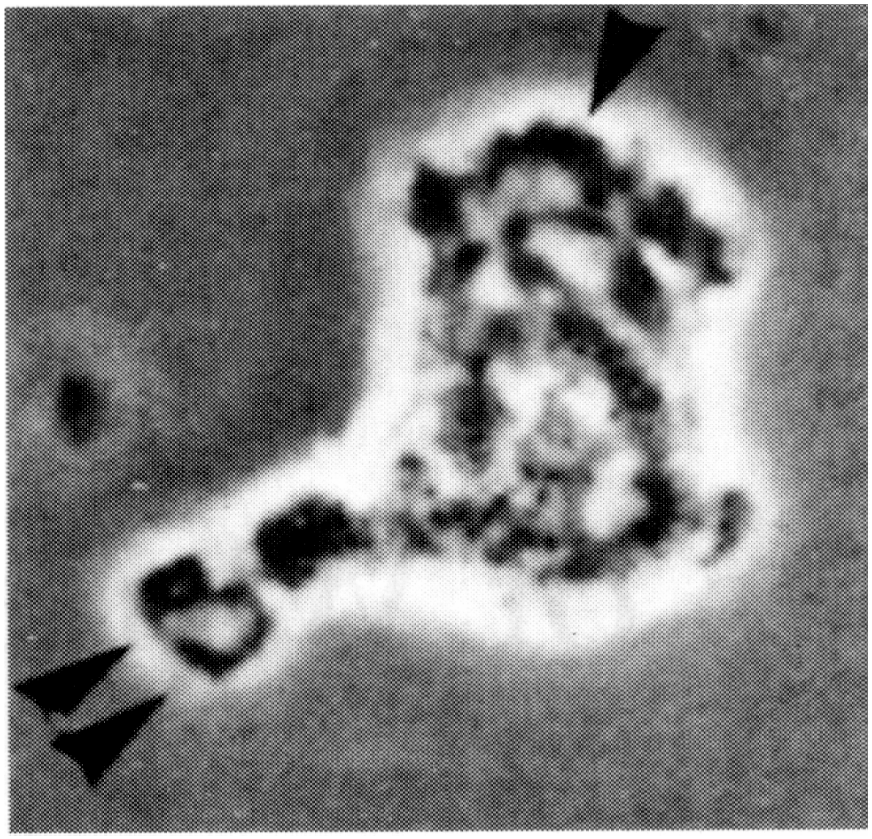


Figure 19. Polarized morphology of human neutrophils. Polarized neutrophils are characterized by the formation of distinct, electron dense regions at opposing ends of cellular migration. The lamellapodium is a flattened membrane that defines the front of migrating cells that is ruffled and often decorated with small narrow projections called microspikes. This region of membrane houses the signaling effectors that transduce movement by variously engaging cytoplasmic proteins that differentially modify the actin-enriched cortical cytoskeleton resident beneath the lamellapod membrane. The trailing edge of cellular migration is typified by a knob-like structure, the uropod, whose function in chemotaxis is not yet determined. Single arrowhead denotes the protruding lamellar membrane present at the leading edge of cellular migration, while the trailing edge, or uropod, is depicted by the double arrowheads. From T.P. Stossel {107}.

activated versus nonactivated neutrophils, the observed inconsistencies in kinetic studies examining the neutrophil polarization process may be explained by measurements made on activated populations misidentified by some investigators as ‘nonactivated.’

The morphological changes that follow cellular activation are well-described and involve modifications of neutrophil plasma membranes and underlying membrane skeletal structure into respective flattened and dense networks at the leading edge of directed migration, while the trailing edge assumes a narrow knob-like morphology (Figure 19). In direct contrast to the observations noted above (Gerisch and Keller, 1981;Davis et al., 1982), some investigators have found that fMLF-stimulated, adherent neutrophils first undergo morphological changes in association with cell-polarization that markedly differ from those listed above within seconds after stimulation with fMLF (Davis et al., 1982;Oliver and Berlin, 1982;Seveau et al., 2001a). It has been suggested that such conflicts may be based on a general failure to correctly identify the cellular activation-state of neutrophil populations prior to their analysis (Servant et al., 1999). Importantly, the timing of secretory vesicle-plasma membrane fusion events relative to onset of polarization is not known. Notably, however, Kobayashi et al. (Kobayashi et al., 1998b;Kobayashi et al., 2000) have shown a direct quantitative correlation between the fMLF-induced cellular activation, the dynamic reorganization of secretory organelles, and the induction of specific downstream effector activities in human neutrophils. Significantly, their data suggest that initial FPR signaling events in resting plasma membranes is a complex process integrating the assembly of cell surface receptor signaling pathways with the secretory properties of neutrophils. Thus, fMLF-directed

polarization and subsequent migration may well proceed through an interdependent and sequential chain of intracellular events involving i) FPR signaling; ii) secretory vesicle mobilization to plasma membranes; and iii) effector response (Jiang et al., 1996;Jiang et al., 1998;Kobayashi et al., 1998a;Robinson et al., 1999;Seguchi and Kobayashi, 2002). These data further indicate that the complex fusion events occurring during the course of secretory vesicle exocytosis may comprise an essential driving force that affects both polarization processes and the mechanics of directed migration. As such, this exocytic event may comprise an essential element in receptor/response regulation.

Proposed Biochemical Standardization of the Neutrophil Activation State

In order for activated cell movement to occur, appropriate signaling pathways must be engaged. This can only occur when plasma membranes are provided access to signaling molecules that transduce cell motility (Funamoto et al., 2002). One remaining question in this regard concerns the cellular state (i.e., non-activated, partially, and fully activated) in which cells are capable of engaging the appropriate pathways that culminate in directed movement. The ability of neutrophils to assume multiple, functionally distinct cellular states in response to agonist exposure may imply that *in vitro* isolated neutrophils are either exquisitely sensitive to environmental change or inherently unstable and undergo spontaneous transitions in cellular activation state. This latter point has been suggested by the discovery of several plasma proteins that actively regulate circulating blood-neutrophils to insure they remain quiescent in the absence of proinflammatory factors (Tschesche et al., 1994;Balke et al., 1995;Haag-Weber and Horl, 1995;Horl, 2002). *In vitro* isolated neutrophils are free from the influence of such proteins and may

therefore spontaneously activate, as discussed in greater detail in Chapter 2. In addition, other variables, including various ambient factors (such as microbial contaminants, temperatures at or above 37ºC, and mechanical perturbation) may be introduced during the course of blood-neutrophil isolation that directly alter the functional state of neutrophils. A standardized set of simple and straight-forward guidelines implemented by laboratories involved in neutrophil research would insure the comparability of results between different laboratories, eliminate assumptions that lead to the misinterpretation of experimental results, and possibly resolve various current controversies in this field of study (see below). Suggested guidelines for evaluation of the neutrophil activation state include the following:

i) Quantitation of the secretory vesicle complement remaining within *in vitro* isolated neutrophils. This test can be performed biochemically within 15-30 minutes following blood cell isolation using ~10^5 cells. If the fraction of intact secretory vesicles in isolated neutrophils is equal to or greater than 60% of the original complement present in circulating neutrophil, no additional testing is necessary, and cells can be considered to occupy a nonactivated cellular state. However if intact secretory vesicles number less than 60% of the original cellular complement, the additional test indicated below must be performed.

ii) Adherence on plastic is a simple, rapid approach to evaluating cellular activation state. This test can be performed within 10 minutes, requires 10^5 cells and the use of only basic materials. Although plastic is a

nonphysiological substrate, it has been used to evaluate neutrophil adherence in numerous studies, so the responsiveness of neutrophils to plastic has been thoroughly characterized (Fehr, 1977;Fehr and Jacob, 1977;Fehr and Dahinden, 1979;Dahinden and Fehr, 1983;Dahinden et al., 1983a;Dahinden et al., 1983b;Ginis and Tauber, 1990). Evaluation of results is based on the principal that the extent of cellular adherence is determined by the plasma membrane expression of cellular adhesion molecules, which is, in turn, determined by the cellular activation state. This principle is only true when gauging cellular adhesion on plastic, as most other substrates serve to activate neutrophils on contact. Results are evaluated by determining the fraction of adherent cells. Neutrophil responsiveness on plastic is typically all-or-nothing. Therefore, the adherent subpopulation will either exceed 70% or comprise less than 4% of the total number of cells plated. With $\geq$ 70% adherence, the population should be considered primed, whereas with < 4% adherence, cells should be considered partially activated. The classification of 'partially activated' is based on the lack of secretory vesicle content (see above) rather than adherence results. The value of this approach as a reliable indicator of the neutrophil activation state is further discussed in Chapter 3, which demonstrates a positive relationship between extent of cellular adherence on plastic and induction of superoxide production.

The current literature is rendered ambiguous by three pervasive assumptions made by some investigators in this field of research. First, there is a general failure of studies to confirm the nonactivated state of neutrophils prior to experimentation. Hence, a standardized biochemical approach allowing quantitative confirmation of *in vitro* isolated neutrophil will prevent the misidentification of neutrophils and thus insure reliable study of the phenotypic transition from the resting to primed state. Because different laboratories use tailored methodologies regarding the *in vitro* isolation of neutrophils and subsequent manipulations, this simplistic confirmatory approach would serve to standardize published data by enforcing a biochemical definition for the nonactivated state.

The rapid kinetics and sensitivity of secretory vesicle mobilization in response to various environmental perturbations is well established (Borregaard et al., 1987). Such studies indicate a high degree of variably in secretory vesicle loss during neutrophil-isolation from whole blood (Figures 10 and 11) and in response to slight *in vitro* manipulations (Borregaard et al., 1987). The unpredictable dynamic behavior of this organelle and its association with the functional conversion of human neutrophils thus emphasizes the need for confirmatory analysis to insure that cells are correctly designated as nonactivated versus primed. In particular, this issue may underlie the following controversial observations:

i) The effector potential (i.e., the capacity of cells to functionally respond to agonist stimulation) of primed neutrophils is found to be inherently variable and, perhaps even unstable, varying markedly in response to

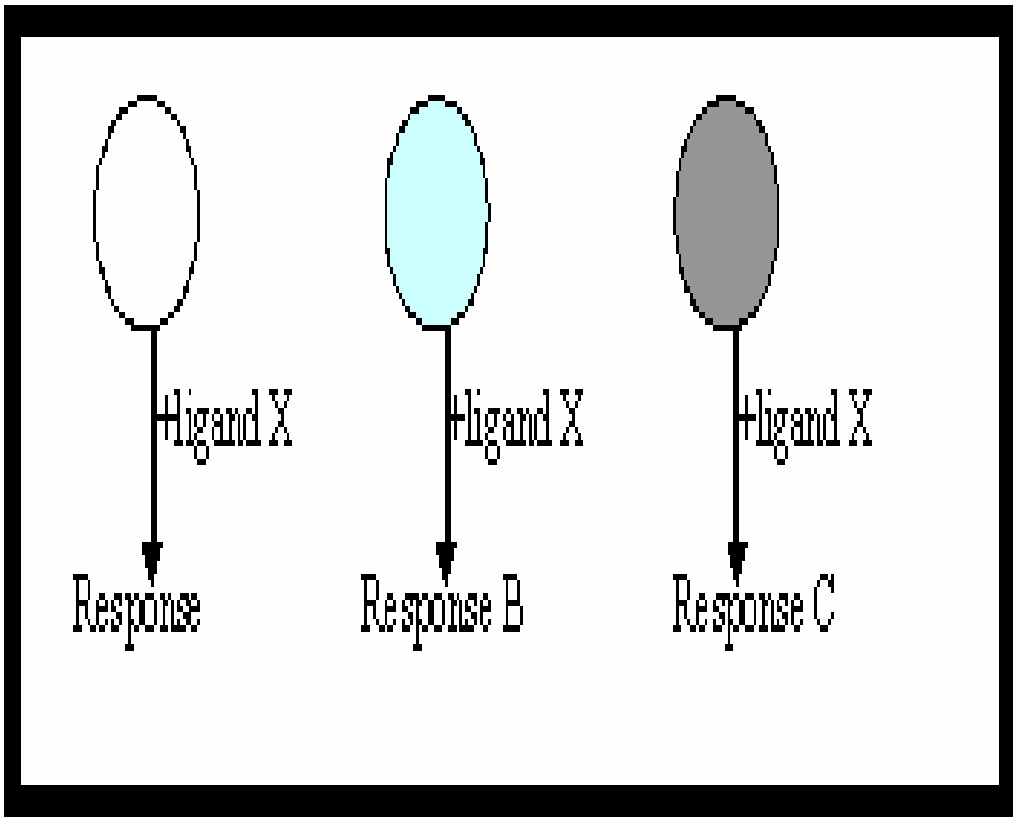


Figure 20. Proposed basis underlying the observed response-variation of resting human neutrophils following stimulation with a specific agonist. Circles: (three) distinct populations of in vitro isolated neutrophils, each obtained from different labs and empirically evaluated to be non-activated. The proportion of spontaneously primed cells in each population is depicted in progressively darker shades. Following stimulation with ligand X, populations vary significantly in responsiveness, as indicated by A, B, C.

different priming-agents (Koenderman et al., 1989;O'Flaherty et al., 1990;McClenahan et al., 2002). In addition, it has been observed that the effector responses of primed versus non-activated neutrophils vary from indistinguishable to markedly different (McColl et al., 1990;Bauldry et al., 1991;Yasui et al., 1992;Durstin et al., 1993;Klein et al., 1995) and that neutrophils can demonstrate various degrees of priming, as judged by effector activity (Verghese et al., 1986;Truett, III et al., 1988). For example, exposure of a primed population to activating ligand will generally promote extensive degranulation and microbicidal activity, whereas the same ligand will initiate effector activities in non-activated populations that are distinctly non-microbicidal (Haribabu et al., 2000). Thus, heterogeneous populations of primed and non-activated phenotypes would be expected to vary, perhaps unstably, in their effector responses in a manner dependent upon the relative ratio of non-activated and primed cells (Fig 20). Such mixed populations of neutrophils, designated empirically as nonactivated, may account for observed inconsistencies (Verghese et al., 1986;Truett, III et al., 1988), thus emphasizing the need for confirmatory measures when evaluating the neutrophil activation state.

ii) Similar concentrations of identical ligand induce markedly different effector responses following various forms of *in vitro* manipulation, including the so-called “aging-effects” displayed by cells after prolonged incubation at room temperature (Dahlgren, 1990) and matrix-dependent

degranulation-effects (Borgquist et al., 2002). The latter, matrix-dependent degranulation-effects, are similar to previous work showing an adherence-associated “priming-effect” when non-activated populations are allowed to spread onto nylon-fibers (Clifford et al., 1984), implying that observed matrix effects (Borgquist et al., 2002) do not bypass the graded paradigm discussed in Figure 12 and are likely due to either the spontaneous or active upregulation of the secretory compartment in these cells. However, both approaches (Dahlgren, 1990;Borgquist et al., 2002) require that cells be extensively manipulated *in vitro* prior to examination, and thus spontaneous priming may be a factor in these areas of study. Furthermore, both types of activation contribute to formation of heterogeneous populations of variously primed neutrophils, empirically designated as nonactivated by some investigators.

iii) Some priming agents are capable of inducing microbicidal activity at ligand concentrations previously described as sub-threshold (Smith et al., 1985;Borgquist et al., 2002). Confirmatory evidence is especially crucial in these studies, given the inherently graded and rapidly inducible nature of neutrophil activation ((Pike et al., 1986;Nusse and Lindau, 1988;Uhing et al., 1989;O'Flaherty et al., 1990;Ortmeyer and Mohsenin, 1993); Fig 12). Arguably, the confounding and controversial findings cited above (i-iii) could have been largely avoided if the above-mentioned guidelines for

defining the nonactivated state of appropriately designated populations had been employed.

A second assumption made by some investigators in the field of neutrophil research pertains to the contribution of secretory vesicle exocytosis during neutrophil chemotaxis. Because secretory vesicles are derived from plasma membranes, this subcellular fraction is incorrectly viewed by many investigators as a subset of recycling endosomes having physical and biochemical properties that are essentially indistinguishable from plasma membranes. Secretory vesicles are, instead, a stable (non-recycling) granule subpopulation with physical and biochemical properties distinct from plasma membranes. Although it is widely held that secretory-plasma membrane organelle fusion events confer partial activation, the kinetics of this process relative to that of directed migration have not been studied. Importantly studies in this regard are necessary in order to evaluate the true effector potential (i.e., ability of cells to respond functionally in response to agonist stimulation) of resting plasma membranes, which must be determined before the mechanistic basis of neutrophil activation can be resolved. This would entail application of the above guidelines to define in practical terms the biochemical features expected of ‘nonactivated’ neutrophils.

The third widely held assumption in the field of neutrophil research is the concept that nonactivated plasma membranes are innately capable of promoting functional conversion of resting neutrophil in the absence of secretory vesicle fusion. On the contrary, studies by Kobayashi et al (Kobayashi et al., 1998a) have indicated that resting plasma membranes are utterly incapable of supporting superoxide generation in the

absence of secretory vesicle fusion. Therefore it is unlikely that nonactivated plasma membranes are capable of transducing signals that culminate in motility, apart from their fusion with secretory vesicles. Because studies in the field of neutrophil chemotaxis have generally neglected to confirm the activation state of the neutrophil populations under analysis, the inherent effector potential of quiescent membranes cannot be determined in light of currently published studies. This point is crucial given that studies examining migration efficiency in the absence of secretory-plasma membrane fusion events in biochemically confirmed non-activated human neutrophils are not included in the published literature. The importance of this issue is indicated by the essential nature of secretory vesicle upregulation to the functional conversion of neutrophils. Indirect evidence is available that suggests the importance of secretory vesicle exocytosis in neutrophil polarization. For example, the microtubule cytoskeleton has been implicated in secretory vesicle exocytosis. This, coupled with widespread evidence that an intact microtubule cytoskeleton facilitates orientation and thus initial polarization of chemoattractant-stimulated cells (Bershadsky et al., 1991;Rodionov et al., 1993;Bershadsky and Vasiliev, 1993), could implicate secretory vesicle-plasma membrane fusion in cell polarization. Though numerous studies have examined the impact of microtubule inhibitors on various functional responses of isolated human neutrophils, the potential importance of secretory vesicle-plasma membrane fusion events to the morphological structures adopted by activated cells remain speculative because of a general failure to seek biochemical-confirmation of designated nonactivated populations.

The relative distribution and accessibility of signaling components in nonactivated versus primed and fully activated plasma membranes in relation to fMLF-directed chemotaxis also remains unknown. Importantly intact secretory vesicles associate with signaling pathways that are absent in resting plasma membranes. Upregulation of secretory vesicles could therefore confer on plasma membranes new signaling properties and suggests that accessibility of effector molecules to plasma membranes can vary with cellular activation state and, hence, the extent of secretory vesicle upregulation (Bloch et al., 2001). For example, FPR occupancy in nonactivated neutrophils (in which the majority of secretory vesicles remain intact) may produce signaling cascades that markedly differ in functional outcome relative to FPR occupancy in partially activated (in which secretory vesicles are partially upregulated) or fully activated plasma neutrophils (in which secretory vesicles are fully upregulated) (see Chapter 3). This emphasizes the importance of biochemical standardization of the neutrophil activation state, especially in view of observations detailing (i) marked differences in plasma membrane composition and effector capacity between non-activated and primed neutrophils (Borregaard et al., 1987;Borregaard et al., 1990;Sengelov et al., 1993a;Borregaard et al., 1994) and (ii) cell surface delivery of receptor or effector proteins like FPR that are enriched in secretory vesicles (Sengelov et al., 1994a). Most importantly, clarification of this issue would afford a greater insight into the regulation of neutrophil activation *in vivo* that could lead to novel methods of therapeutic intervention for the treatment of various inflammatory disorders. Adoption of a prescribed, standardized method for the quantitation of secretory vesicles within *in vitro* isolated populations (see above) could eliminate ambiguities

related to qualitative assessment of the neutrophil activation state and thus clarify the effector potential of resting plasma membranes. In addition, determining the:

i) kinetics of secretory-plasma membrane fusion relative to the induction of mechanical events that affect directed migration.

ii) spatial proximity of fusion events relative to the leading edge of migration.

could contribute to current understanding of the biology of directed migration and may in turn suggest novel therapeutic approaches for the treatment of inflammation-related disorders.

Clinical Significance in Relation to Congenital Disorders of Blood-Neutrophils. Although neutrophil physiology is exceptionally complex, neutrophil activation appears to be a very well regimented process that is dependent upon the sequential fusion of secretory and granular fractions with non-activated and partially activated plasma membranes, respectively (Bentwood and Henson, 1980;Borregaard et al., 1987;Borregaard et al., 1990;Sengelov et al., 1993b;Ligeti and Mocsai, 1999). A differential signaling threshold for neutrophil chemotaxis and cytotoxicity is well documented (Honeycutt and Niedel, 1986;Truett, III et al., 1988;Uhing et al., 1989) and indicates that the mobilization of secretory and granular fractions is interdependent (Nusse and Lindau, 1988;Sengelov et al., 1993b;Ligeti and Mocsai, 1999). Indeed, while the signaling threshold is markedly lower for the mobilization of secretory vesicles relative to the primary and secondary granule subsets, their strict temporal regulation is suggestive of a complexity that is not yet fully appreciated (Ligeti and Mocsai, 1999), especially in terms of the relative interdependence

of their exocytic behavior. In particular, the marked phenotypic conversion induced by the fusion of secretory vesicle and plasma membrane organelles (Borregaard et al., 1994) suggest a mechanism whereby quiescent cells maintain a resting phenotype *in vivo*. Indeed, the sequestration of activated, PML-enriched membrane in resting cells is in keeping with evidence of the inordinately high constitutive activity of FPR (Wenzel-Seifert et al., 1998b;Seifert and Wenzel-Seifert, 2001). According to our hypothesis, resting neutrophils address this problem by differentially coupling various FPR pools with functionally distinct domains. In this scenario, the receptor pools present in resting plasma membranes are rendered inactive by the absence of certain downstream effectors, while the PML-enriched FPR is safely sequestered within intact secretory vesicles. Thus, the vesicular segregation of FPR pools represents a physiological mechanism whereby cells can inhibit inappropriate receptor activation by regulating their access to various downstream effectors. This concept has important therapeutic implications.

Significantly, current, longstanding therapeutic approaches that employ either microtubule antagonists for immunosuppression or ascorbic acid to normalize microtubule polymerization (and thus restore neutrophil activation in patients with, for example, Chediak-Hagashi Syndrome) may act principally at the level of secretory vesicle-plasma membrane fusion. Both treatments significantly and conversely affect neutrophil activation and effector function *in vivo* (Boxer et al., 1978;Boxer et al., 1979;Pryzwansky et al., 1985;Dahlgren et al., 1987;Cronstein et al., 1995). While Chediak-Hagashi Syndrome is attributed to a marked deficiency in neutrophil degranulation, (Dell'Angelica et al., 2000) patient neutrophils are also deficient in

effector functions characteristic of the primed cellular state, suggesting an impairment of secretory vesicle-plasma membrane fusion (Boxer et al., 1979;Colgan et al., 1992). Thus, it is likely that inhibitory affects on secretory-plasma membrane fusion events also contribute to the hallmark characteristics of neutrophil dysfunction observed in this sydrome (see below). This difference would also support a facilitating role for secretory endosomes in subsequent granular fusion events.

Although the scope of this investigation is primarily concerned with the study of secretory vesicle mobilization within *in vitro* isolated neutrophils (Chapters 2-3), the interdependence of distinct granule subsets, in terms of their concomitant exocytic behavior, is of related interest. Such interdependence of the primed and fully activated states bears directly on the immunotherapeutic potential of treatments that target leukocyte function by:

i) Affording a basis for the development of a new class of pharmacological agents inhibiting the foremost and facilitating events of neutrophil activation;

ii) Providing additional insights into the mechanistic basis of how commonly used immunotherapeutic agents modulate disease outcome, *in vivo*.

The latter point is based on the activation potential (i.e., the ability of cells to acquire microbicidal function in response to agonist stimulation) of neutrophils isolated from patients afflicted with the Chediak-Higashi syndrome (Boxer et al., 1979;Colgan et al., 1992). Given the significant pool of chemoattractant receptors within secretory endosomes (Sengelov et al., 1994a) and their selective, rapid upregulation during

chemotaxis (Borregaard et al., 1987), the impairment of chemotaxis in neutrophils from patients with Chediak-Hagashi syndrome suggests that the scope of neutrophil dysfunction in these patients may involve defective secretory vesicle-plasma membrane fusion events. Accordingly, the restitution of microtubule function in patient neutrophils observed following administration of ascorbic acid (Boxer et al., 1979) may be directly attributable to the restoration of secretory vesicle mobilization in these patients if, in fact, the graded activation state discussed in Figure 12 is derived from a series of temporally distinct, interdependent fusion events. It follows that the nature of neutrophil activation and their unique activation requirements could allow for the development of a class of drugs that disable neutrophils before they have a chance to activate. Importantly, the study of secretory vesicle-plasma membrane fusion could contribute to the identification of lineage-specific effector or signaling proteins required during the initial stages of neutrophil activation that are amenable to targeted inactivation by therapeutic agents. For example, membrane-permeant agents that are made temporarily soluble in plasma by a degradable hydrophilic casing could rapidly equilibrate in blood and subsequently enter blood neutrophils to disable intracellular effector proteins required during the initial stages of their activation. The unique activation properties of neutrophils (relative to other blood-leukocytes) offer a basis for the selectivity of such agents, and thus a means to selectively turn-off blood-neutrophils. This form of *selective* therapeutic intervention has several advantages over drugs that target signaling processes fundamental to all somatic cells. These latter agents are inherently nonspecific, can affect virtually any

patient cell-type, and thus lead to unpredictable side effects (Sieper and Braun, 2001;McMurray and Hardy, 2002).

This investigation supports an approach to intervention that acts on circulating, quiescent cells, effectively disarming them so that they cannot activate. Given the finding that quiescent neutrophils are likely to be compromised in their ability to extravasate apart from secretory vesicle-plasma membrane fusion (Borregaard et al., 1990;Borregaard et al., 1994). Such an approach could promote a significant decrease in the numbers of inflammatory cells capable of entering extravascular sites of acute or chronic inflammation. Moreover, because neutrophils contribute extensively to inflammatory cascades by releasing an impressive array of mediators, including cytokines (Cassatella, 1999;Hattar et al., 2006;Robertson et al., 2006), chemokines (Fredriksson et al., 2002;Schroder et al., 2005;Jozsef et al., 2006;Pellme et al., 2006), platelet activating factor (Haroldsen et al., 1987), eicosanoids (Nagano et al., 2002), and interferons (Taborsky and Dolnik, 1977), this therapeutic approach may prove an exceptionally effective anti-inflammatory strategy (Witko-Sarsat et al., 2000). Thus, the targeted approach to therapeutic intervention suggested here would potentially minimize side-effects in dosage-independent manner, thereby affording maximal down regulation of effector cells without subjecting patients to the unpredictable side effects associated with other conventional approaches.

Opportunistic Disease and Treatment Strategies.

Although exploitation of the unique physiological requirements of neutrophils does not contribute to treatment of immune-compromised patients, the study of specific

opportunistic pathogens has led to important insights regarding the adaptive capacity and versatility of plasma membrane function in microbial pathogenesis. This final section will focus on major issues involved in the treatment of opportunistic infection and discuss a specific case study that uniquely correlates possible alterations in plasma membrane lipid biochemistry with modulation of disease virulence in the fungal opportunist, *Candida glabrata*.

Challenges in providing effective therapeutic treatment are significantly enhanced in the immune-compromised due to the expanded number and diversity of potential disease-causing microorganisms that affect this patient population. Neutrophil function is usually normal is in such patients, but the number of circulating leukocytes is significantly decreased, leading to an inability to control and resolve infection. Opportunistists that normally colonize specific anatomical sites become capable of uncontrolled growth and local infection that can spread regionally and/or systemically to become life-threatening (Whimbey et al., 1986;Eng et al., 1986;Witt et al., 1987;Whimbey et al., 1987;Krumholz et al., 1989;Kiehn and Armstrong, 1990;Tumbarello et al., 1995;Kirkpatrick et al., 1997;Kumashi et al., 2005). In many cases, opportunistic infections are resistant to conventional therapeutics that control and eliminate true pathogens (Goldmann et al., 1999;Currier, 2000;Bayat et al., 2003;Nakamoto et al., 2004;Scott et al., 2005). What is more, most currently prescribed treatments specifically targeting opportunistic pathogens are ineffectual and associated with toxic side-effects (Denning et al., 1997;Pelletier et al., 2000).

The final chapter of this dissertation, Chapter 5, describes an additional complication associated with some fungal opportunistic infections of the *Candida* species, *glabrata*. In this disease, yeast-forms normally incapable of infecting certain anatomical sites are found to persist within such areas and genetically adapt to site-specific microenvironmental constraints that normally act to prevent microbial growth and infection. The genetically altered organisms described in this study acquire a severely limited, rather than expanded, growth capacity that, *in vitro*, is characterized by their inability to survive on the nutrient enriched media normally used for the cultivation of fungi. As a result, an important limitation of current nutrient-based protocols used for the targeted isolation of opportunistic fungal strains is realized. This work thus highlights a novel strategy of fungal infection whereby individual yeasts presumably alter their genetic structure, gene expression, mRNA transcriptional profile, and cellular protein production. These alterations modify normal plasma membrane function and disable conventional cellular metabolic function and homeostatic mechanisms in favor of an enhanced cellular requirement for fatty acid and cholesterol in the form of bile that is both noxious and lethal to other more robust and adaptable strains.

LITERATURE CITED

Aderem A. 1992. Signal transduction and the actin cytoskeleton: the roles of MARCKS and profilin. Trends Biochem Sci 17:438-443.

Afeltra A, Caccavo D, Ferri GM, Addessi MA, De Rosa FG, Amoroso A, and Bonomo L. 1997. Expression of lactoferrin on human granulocytes: analysis with polyclonal and monoclonal antibodies. Clin Exp Immunol 109:279-285.

Ahmed SN, Brown DA, and London E. 1997. On the origin of sphingolipid/cholesterol-rich detergent-insoluble cell membranes: physiological concentrations of cholesterol and sphingolipid induce formation of a detergent-insoluble, liquid-ordered lipid phase in model membranes. Biochemistry 36:10944-10953.

Allen WE, Zicha D, Ridley AJ, and Jones GE. 1998. A role for Cdc42 in macrophage chemotaxis. J Cell Biol 141:1147-1157.

Allenspach EJ, Cullinan P, Tong J, Tang Q, Tesciuba AG, Cannon JL, Takahashi SM, Morgan R, Burkhardt JK, and Sperling AI. 2001. ERM-dependent movement of CD43 defines a novel protein complex distal to the immunological synapse. Immunity 15:739-750.

Almers W. 1990. Exocytosis. Annu Rev Physiol 52:607-624.

Altman A, Kaminski S, Busuttil V, Droin N, Hu J, Tadevosyan Y, Hipskind RA, and Villalba M. 2004. Positive feedback regulation of PLCgamma1/Ca(2+) signaling by PKCtheta in restimulated T cells via a Tec kinase-dependent pathway. Eur J Immunol 34:2001-2011.

Aman MJ and Ravichandran KS. 2000. A requirement for lipid rafts in B cell receptor induced Ca(2+) flux. Curr Biol 10:393-396.

Anderson RG. 1998. The caveolae membrane system. Annu Rev Biochem 67:199-225.

Arvanitis DN, Min W, Gong Y, Heng YM, and Boggs JM. 2005. Two types of detergent-insoluble, glycosphingolipid/cholesterol-rich membrane domains from isolated myelin. J Neurochem 94:1696-1710.

Bainton DF. 1999. Distinct granule populations in human neutrophils and lysosomal organelles identified by immuno-electron microscopy. J Immunol Methods 232:153-168.

Bainton DF, Ullyot JL, and Farquhar MG. 1971. The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. J Exp Med 134:907-934.

Baird B, Sheets ED, and Holowka D. 1999. How does the plasma membrane participate in cellular signaling by receptors for immunoglobulin E? Biophys Chem 82:109-119.

Balazovich KJ, Fernandez R, Hinkovska-Galcheva V, Suchard SJ, and Boxer LA. 1996. Transforming growth factor-beta1 stimulates degranulation and oxidant release by adherent human neutrophils. J Leukoc Biol 60:772-777.

Balboa MA and Insel PA. 1995. Nuclear phospholipase D in Madin-Darby canine kidney cells. Guanosine 5'-O-(thiotriphosphate)-stimulated activation is mediated by RhoA and is downstream of protein kinase C. J Biol Chem 270:29843-29847.

Balke N, Holtkamp U, Horl WH, and Tschesche H. 1995. Inhibition of degranulation of human polymorphonuclear leukocytes by complement factor D. FEBS Lett 371:300-302.

Balla T. 2001. Pharmacology of phosphoinositides, regulators of multiple cellular functions. Curr Pharm Des 7:475-507.

Balla T. 2005. Inositol-lipid binding motifs: signal integrators through protein-lipid and protein-protein interactions. J Cell Sci 118:2093-2104.

Barabe F, Pare G, Fernandes MJ, Bourgoin SG, and Naccache PH. 2002b. Cholesterol-modulating agents selectively inhibit calcium influx induced by chemoattractants in human neutrophils. J Biol Chem 277:13473-13478.

Barabe F, Rollet-Labelle E, Gilbert C, Fernandes MJ, Naccache SN, and Naccache PH. 2002a. Early events in the activation of Fc gamma RIIA in human neutrophils: stimulated insolubilization, translocation to detergent-resistant domains, and degradation of Fc gamma RIIA. J Immunol 168:4042-4049.

Barenholz Y. 2004. Sphingomyelin and cholesterol: from membrane biophysics and rafts to potential medical applications. Subcell Biochem 37:167-215.

Barkalow K, Witke W, Kwiatkowski DJ, and Hartwig JH. 1996. Coordinated regulation of platelet actin filament barbed ends by gelsolin and capping protein. J Cell Biol 134:389-399.

Barnett-Norris J, Lynch D, and Reggio PH. 2005. Lipids, lipid rafts and caveolae: their importance for GPCR signaling and their centrality to the endocannabinoid system. Life Sci 77:1625-1639.

Bastiaanse EM, Atsma DE, Van der Valk LJ, and Van der LA. 1995. Metabolic inhibition of cardiomyocytes causes an increase in sarcolemmal fluidity which may be due to loss of cellular cholesterol. Arch Biochem Biophys 319:350-354.

Bauldry SA, Bass DA, Cousart SL, and McCall CE. 1991. Tumor necrosis factor alpha priming of phospholipase D in human neutrophils. Correlation between phosphatidic acid production and superoxide generation. J Biol Chem 266:4173-4179.

Bayat A, Shaaban H, Dodgson A, and Dunn KW. 2003. Implications for Burns Unit design following outbreak of multi-resistant Acinetobacter infection in ICU and Burns Unit. Burns 29:303-306.

Becamel C, Alonso G, Galeotti N, Demey E, Jouin P, Ullmer C, Dumuis A, Bockaert J, and Marin P. 2002. Synaptic multiprotein complexes associated with 5-HT(2C) receptors: a proteomic approach. EMBO J 21:2332-2342.

Becher A and McIlhinney RA. 2005. Consequences of lipid raft association on G-protein-coupled receptor function. Biochem Soc Symp151-164.

Becker KP and Hannun YA. 2005. Protein kinase C and phospholipase D: intimate interactions in intracellular signaling. Cell Mol Life Sci 62:1448-1461.

Belisle B and Abo A. 2000. N-Formyl peptide receptor ligation induces rac-dependent actin reorganization through Gbeta gamma subunits and class Ia phosphoinositide 3-kinases. J Biol Chem 275:26225-26232.

Benard V, Bohl BP, and Bokoch GM. 1999. Characterization of rac and cdc42 activation in chemoattractant-stimulated human neutrophils using a novel assay for active GTPases. J Biol Chem 274:13198-13204.

Bennett TA, Foutz TD, Gurevich VV, Sklar LA, and Prossnitz ER. 2001. Partial phosphorylation of the N-formyl peptide receptor inhibits G protein association independent of arrestin binding. J Biol Chem 276:49195-49203.

Bentwood BJ and Henson PM. 1980. The sequential release of granule constitutents from human neutrophils. J Immunol 124:855-862.

Bergmann JE, Kupfer A, and Singer SJ. 1983. Membrane insertion at the leading edge of motile fibroblasts. Proc Natl Acad Sci U S A 80:1367-1371.

Berkow RL, Dodson RW, and Kraft AS. 1987. The effect of a protein kinase C inhibitor, H-7, on human neutrophil oxidative burst and degranulation. J Leukoc Biol 41:441-446.

Bershadsky AD, Balaban NQ, and Geiger B. 2003. Adhesion-dependent cell mechanosensitivity. Annu Rev Cell Dev Biol 19:677-695.

Bershadsky AD, Vaisberg EA, and Vasiliev JM. 1991. Pseudopodial activity at the active edge of migrating fibroblast is decreased after drug-induced microtubule depolymerization. Cell Motil Cytoskeleton 19:152-158.

Bershadsky AD and Vasiliev JM. 1993. Mechanisms of regulation of pseudopodial activity by the microtubule system. Symp Soc Exp Biol 47:353-373.

Bertram TA and Coignoul FL. 1982. Morphometry of equine neutrophils isolated at different temperatures. Vet Pathol 19:534-543.

Bevers EM, Comfurius P, and Zwaal RF. 1983. Changes in membrane phospholipid distribution during platelet activation. Biochim Biophys Acta 736:57-66.

Bian J, Cui J, and McDonald TV. 2001. HERG K(+) channel activity is regulated by changes in phosphatidyl inositol 4,5-bisphosphate. Circ Res 89:1168-1176.

Billah MM, Eckel S, Mullmann TJ, Egan RW, and Siegel MI. 1989. Phosphatidylcholine hydrolysis by phospholipase D determines phosphatidate and diglyceride levels in chemotactic peptide-stimulated human neutrophils. Involvement of phosphatidate phosphohydrolase in signal transduction. J Biol Chem 264:17069-17077.

Bishop WR and Bell RM. 1988. Assembly of phospholipids into cellular membranes: biosynthesis, transmembrane movement and intracellular translocation. Annu Rev Cell Biol 4:579-610.

Bjerrum OW and Borregaard N. 1989. Dual granule localization of the dormant NADPH oxidase and cytochrome b559 in human neutrophils. Eur J Haematol 43:67-77.

Blanc C, Charette S, Cherix N, Lefkir Y, Cosson P, and Letourneur F. 2005. A novel phosphatidylinositol 4,5-bisphosphate-binding domain targeting the Phg2 kinase to the membrane in Dictyostelium cells. Eur J Cell Biol 84:951-960.

Bloch W, Fan Y, Han J, Xue S, Schoneberg T, Ji G, Lu ZJ, Walther M, Fassler R, Hescheler J, Addicks K, and Fleischmann BK. 2001. Disruption of cytoskeletal integrity impairs Gi-mediated signaling due to displacement of Gi proteins. J Cell Biol 154:753-761.

Blonder J, Hale ML, Lucas DA, Schaefer CF, Yu LR, Conrads TP, Issaq HJ, Stiles BG, and Veenstra TD. 2004. Proteomic analysis of detergent-resistant membrane rafts. Electrophoresis 25:1307-1318.

Bockaert J, Marin P, Dumuis A, and Fagni L. 2003. The 'magic tail' of G protein-coupled receptors: an anchorage for functional protein networks. FEBS Lett 546:65-72.

Bokoch GM. 1995. Chemoattractant signaling and leukocyte activation. Blood 86:1649-1660.

Bommakanti RK, Dratz EA, Siemsen DW, and Jesaitis AJ. 1994. Characterization of complex formation between Gi2 and octyl glucoside solubilized neutrophil N-formyl

peptide chemoattractant receptor by sedimentation velocity. Biochim Biophys Acta 1209:69-76.

Borgquist JD, Quinn MT, and Swain SD. 2002. Adhesion to extracellular matrix proteins modulates bovine neutrophil responses to inflammatory mediators. J Leukoc Biol 71:764-774.

Borregaard N. 1996. Current concepts about neutrophil granule physiology. Curr Opin Hematol 3:11-18.

Borregaard N, Christensen L, Bejerrum OW, Birgens HS, and Clemmensen I. 1990. Identification of a highly mobilizable subset of human neutrophil intracellular vesicles that contains tetranectin and latent alkaline phosphatase. J Clin Invest 85:408-416.

Borregaard N, Kjeldsen L, Lollike K, and Sengelov H. 1995. Granules and secretory vesicles of the human neutrophil. Clin Exp Immunol 101 Suppl 1:6-9.

Borregaard N, Kjeldsen L, Rygaard K, Bastholm L, Nielsen MH, Sengelov H, Bjerrum OW, and Johnsen AH. 1992. Stimulus-dependent secretion of plasma proteins from human neutrophils. J Clin Invest 90:86-96.

Borregaard N, Kjeldsen L, Sengelov H, Diamond MS, Springer TA, Anderson HC, Kishimoto TK, and Bainton DF. 1994. Changes in subcellular localization and surface expression of L-selectin, alkaline phosphatase, and Mac-1 in human neutrophils during stimulation with inflammatory mediators. J Leukoc Biol 56:80-87.

Borregaard N, Miller LJ, and Springer TA. 1987. Chemoattractant-regulated mobilization of a novel intracellular compartment in human neutrophils. Science 237:1204-1206.

Boulay F, Tardif M, Brouchon L, and Vignais P. 1990. Synthesis and use of a novel N-formyl peptide derivative to isolate a human N-formyl peptide receptor cDNA. Biochem Biophys Res Commun 168:1103-1109.

Bowman EP, Uhlinger DJ, and Lambeth JD. 1993. Neutrophil phospholipase D is activated by a membrane-associated Rho family small molecular weight GTP-binding protein. J Biol Chem 268:21509-21512.

Bowman EP, Uhlinger DJ, and Lambeth JD. 1995. Neutrophil phospholipase D: inhibition by Rho-GDP dissociation inhibitor and stimulation by small GTPase GDP dissociation stimulator. Methods Enzymol 256:246-256.

Boxer LA, Albertini DF, Baehner RL, and Oliver JM. 1979. Impaired microtubule assembly and polymorphonuclear leucocyte function in the Chediak-Higashi syndrome correctable by ascorbic acid. Br J Haematol 43:207-213.

Boxer LA, Allen JM, Watanabe AM, Besch HR, Jr., and Baehner RL. 1978. Role of microtubules in granulocyte adherence. Blood 51:1045-1050.

Boxer LA, Haak RA, Yang HH, Wolach JB, Whitcomb JA, Butterick CJ, and Baehner RL. 1982. Membrane-bound lactoferrin alters the surface properties of polymorphonuclear leukocytes. J Clin Invest 70:1049-1057.

Brady AE and Limbird LE. 2002. G protein-coupled receptor interacting proteins: emerging roles in localization and signal transduction. Cell Signal 14:297-309.

Bray D, Heath J, and Moss D. 1986. The membrane-associated 'cortex' of animal cells: its structure and mechanical properties. J Cell Sci Suppl 4:71-88.

Bretscher A. 1991. Microfilament structure and function in the cortical cytoskeleton. Annu Rev Cell Biol 7:337-374.

Bretscher A, Chambers D, Nguyen R, and Reczek D. 2000. ERM-Merlin and EBP50 protein families in plasma membrane organization and function. Annu Rev Cell Dev Biol 16:113-143.

Bretscher A, Edwards K, and Fehon RG. 2002. ERM proteins and merlin: integrators at the cell cortex. Nat Rev Mol Cell Biol 3:586-599.

Bretscher A and Weber K. 1980. Fimbrin, a new microfilament-associated protein present in microvilli and other cell surface structures. J Cell Biol 86:335-340.

Bromley SK, Burack WR, Johnson KG, Somersalo K, Sims TN, Sumen C, Davis MM, Shaw AS, Allen PM, and Dustin ML. 2001. The immunological synapse. Annu Rev Immunol 19:375-396.

Brown DA and London E. 1997. Structure of detergent-resistant membrane domains: does phase separation occur in biological membranes? Biochem Biophys Res Commun 240:1-7.

Brown DA and London E. 2000. Structure and function of sphingolipid- and cholesterol-rich membrane rafts. J Biol Chem 275:17221-17224.

Brown FD, Rozelle AL, Yin HL, Balla T, and Donaldson JG. 2001. Phosphatidylinositol 4,5-bisphosphate and Arf6-regulated membrane traffic. J Cell Biol 154:1007-1017.

Brown HA, Gutowski S, Moomaw CR, Slaughter C, and Sternweis PC. 1993. ADP-ribosylation factor, a small GTP-dependent regulatory protein, stimulates phospholipase D activity. Cell 75:1137-1144.

Brzostowski JA and Kimmel AR. 2001. Signaling at zero G: G-protein-independent functions for 7-TM receptors. Trends Biochem Sci 26:291-297.

Bunemann M and Hosey MM. 1999. G-protein coupled receptor kinases as modulators of G-protein signalling. J Physiol 517 (Pt 1):5-23.

Buss JE, Mumby SM, Casey PJ, Gilman AG, and Sefton BM. 1987. Myristoylated alpha subunits of guanine nucleotide-binding regulatory proteins. Proc Natl Acad Sci U S A 84:7493-7497.

Cabanis A, Gressier B, Brunet C, Dine T, Luyckx M, Cazin M, and Cazin JC. 1996. Effect of the protein kinase C inhibitor GF 109 203X on elastase release and respiratory burst of human neutrophils. Gen Pharmacol 27:1409-1414.

Caccavo D, Garzia P, Sebastiani GD, Ferri GM, Galluzzo S, Vadacca M, Rigon A, Afeltra A, and Amoroso A. 2003. Expression of lactoferrin on neutrophil granulocytes from synovial fluid and peripheral blood of patients with rheumatoid arthritis. J Rheumatol 30:220-224.

Carbajal ME, Manning-Cela R, Pina A, Franco E, and Meza I. 1996. Fibronectin-induced intracellular calcium rise in Entamoeba histolytica trophozoites: effect on adhesion and the actin cytoskeleton. Exp Parasitol 82:11-20.

Caroni P. 2001. New EMBO members' review: actin cytoskeleton regulation through modulation of PI(4,5)P(2) rafts. EMBO J 20:4332-4336.

Carpenter CL, Tolias KF, Van Vugt A, and Hartwig J. 1999. Lipid kinases are novel effectors of the GTPase Rac1. Adv Enzyme Regul 39:299-312.

Cassatella MA. 1999. Neutrophil-derived proteins: selling cytokines by the pound. Adv Immunol 73:369-509.

Cederholm T and Gyllenhammar H. 1999. Impaired granulocyte formylpeptide-induced superoxide generation in chronically ill, malnourished, elderly patients. J Intern Med 245:475-482.

Cereijido M, Valdes J, Shoshani L, and Contreras RG. 1998. Role of tight junctions in establishing and maintaining cell polarity. Annu Rev Physiol 60:161-177.

Chamberlain LH. 2004. Detergents as tools for the purification and classification of lipid rafts. FEBS Lett 559:1-5.

Chen CS, Martin OC, and Pagano RE. 1997. Changes in the spectral properties of a plasma membrane lipid analog during the first seconds of endocytosis in living cells. Biophys J 72:37-50.

Cho H, Hwang JY, Kim D, Shin HS, Kim Y, Earm YE, and Ho WK. 2002. Acetylcholine-induced phosphatidylinositol 4,5-bisphosphate depletion does not cause

short-term desensitization of G protein-gated inwardly rectifying K+ current in mouse atrial myocytes. J Biol Chem 277:27742-27747.

Cho H, Kim YA, Yoon JY, Lee D, Kim JH, Lee SH, and Ho WK. 2005a. Low mobility of phosphatidylinositol 4,5-bisphosphate underlies receptor specificity of Gq-mediated ion channel regulation in atrial myocytes. Proc Natl Acad Sci U S A 102:15241-15246.

Cho H, Lee D, Lee SH, and Ho WK. 2005b. Receptor-induced depletion of phosphatidylinositol 4,5-bisphosphate inhibits inwardly rectifying K+ channels in a receptor-specific manner. Proc Natl Acad Sci U S A 102:4643-4648.

Chou J, Stolz DB, Burke NA, Watkins SC, and Wells A. 2002. Distribution of gelsolin and phosphoinositol 4,5-bisphosphate in lamellipodia during EGF-induced motility. Int J Biochem Cell Biol 34:776-790.

Chung CY, Funamoto S, and Firtel RA. 2001. Signaling pathways controlling cell polarity and chemotaxis. Trends Biochem Sci 26:557-566.

Chung CY, Lee S, Briscoe C, Ellsworth C, and Firtel RA. 2000. Role of Rac in controlling the actin cytoskeleton and chemotaxis in motile cells. Proc Natl Acad Sci U S A 97:5225-5230.

Clapham DE and Neer EJ. 1997. G protein beta gamma subunits. Annu Rev Pharmacol Toxicol 37:167-203.

Clifford DP, Rasp FL, and Repine JE. 1984. Simultaneous measurement of adherence and chemiluminescence by polymorphonuclear leukocytes. Inflammation 8:101-106.

Coates TD, Watts RG, Hartman R, and Howard TH. 1992. Relationship of F-actin distribution to development of polar shape in human polymorphonuclear neutrophils. J Cell Biol 117:765-774.

Cocco L, Gilmour RS, Ognibene A, Letcher AJ, Manzoli FA, and Irvine RF. 1987. Synthesis of polyphosphoinositides in nuclei of Friend cells. Evidence for polyphosphoinositide metabolism inside the nucleus which changes with cell differentiation. Biochem J 248:765-770.

Cockcroft S. 1996. Phospholipid signaling in leukocytes. Curr Opin Hematol 3:48-54.

Cockcroft S and De Matteis MA. 2001. Inositol lipids as spatial regulators of membrane traffic. J Membr Biol 180:187-194.

Cockcroft S and Stutchfield J. 1989. The receptors for ATP and fMetLeuPhe are independently coupled to phospholipases C and A2 via G-protein(s). Relationship between phospholipase C and A2 activation and exocytosis in HL60 cells and human neutrophils. Biochem J 263:715-723.

Cockcroft S, Thomas GM, Fensome A, Geny B, Cunningham E, Gout I, Hiles I, Totty NF, Truong O, and Hsuan JJ. 1994. Phospholipase D: a downstream effector of ARF in granulocytes. Science 263:523-526.

Colgan SP, Blancquaert AM, Thrall MA, and Bruyninckx WJ. 1992. Defective in vitro motility of polymorphonuclear leukocytes of homozygote and heterozygote Chediak-Higashi cats. Vet Immunol Immunopathol 31:205-227.

Comfurius P, Senden JM, Tilly RH, Schroit AJ, Bevers EM, and Zwaal RF. 1990. Loss of membrane phospholipid asymmetry in platelets and red cells may be associated with calcium-induced shedding of plasma membrane and inhibition of aminophospholipid translocase. Biochim Biophys Acta 1026:153-160.

Condliffe AM, Davidson K, Anderson KE, Ellson CD, Crabbe T, Okkenhaug K, Vanhaesebroeck B, Turner M, Webb L, Wymann MP, Hirsch E, Ruckle T, Camps M, Rommel C, Jackson SP, Chilvers ER, Stephens LR, and Hawkins PT. 2005. Sequential activation of class IB and class IA PI3K is important for the primed respiratory burst of human but not murine neutrophils. Blood 106:1432-1440.

Corgan AM, Singleton C, Santoso CB, and Greenwood JA. 2004. Phosphoinositides differentially regulate alpha-actinin flexibility and function. Biochem J 378:1067-1072.

Corrotte M, Chasserot-Golaz S, Huang P, Du G, Ktistakis NT, Frohman MA, Vitale N, Bader MF, and Grant NJ. 2006. Dynamics and function of phospholipase d and phosphatidic Acid during phagocytosis. Traffic 7:365-377.

Corvera S, D'Arrigo A, and Stenmark H. 1999. Phosphoinositides in membrane traffic. Curr Opin Cell Biol 11:460-465.

Costa T, Ogino Y, Munson PJ, Onaran HO, and Rodbard D. 1992. Drug efficacy at guanine nucleotide-binding regulatory protein-linked receptors: thermodynamic interpretation of negative antagonism and of receptor activity in the absence of ligand. Mol Pharmacol 41:549-560.

Cougoule C, Constant P, Etienne G, Daffe M, and Maridonneau-Parini I. 2002. Lack of fusion of azurophil granules with phagosomes during phagocytosis of Mycobacterium smegmatis by human neutrophils is not actively controlled by the bacterium. Infect Immun 70:1591-1598.

Cronstein BN, Molad Y, Reibman J, Balakhane E, Levin RI, and Weissmann G. 1995. Colchicine alters the quantitative and qualitative display of selectins on endothelial cells and neutrophils. J Clin Invest 96:994-1002.

Cunningham CC, Vegners R, Bucki R, Funaki M, Korde N, Hartwig JH, Stossel TP, and Janmey PA. 2001. Cell permeant polyphosphoinositide-binding peptides that block cell motility and actin assembly. J Biol Chem 276:43390-43399.

Currier JS. 2000. Discontinuing prophylaxis for opportunistic infection: guiding principles. Clin Infect Dis 30 Suppl 1:S66-S71.

Dahinden C and Fehr J. 1983. Granulocyte activation by endotoxin. II. Role of granulocyte adherence, aggregation, and effect of cytochalasin B, and comparison with formylated chemotactic peptide-induced stimulation. J Immunol 130:863-868.

Dahinden C, Galanos C, and Fehr J. 1983a. Granulocyte activation by endotoxin. I. Correlation between adherence and other granulocyte functions, and role of endotoxin structure on biologic activity. J Immunol 130:857-862.

Dahinden CA, Fehr J, and Hugli TE. 1983b. Role of cell surface contact in the kinetics of superoxide production by granulocytes. J Clin Invest 72:113-121.

Dahlgren C. 1990. Temporal adaptation of human neutrophil metabolic responsiveness to the peptide formylmethionyl-leucyl phenylalanine: a comparison between human neutrophils and granule-depleted neutrophil cytoplasts. Cell Biochem Funct 8:57-64.

Dahlgren C, Norberg B, and Eriksson S. 1987. The adherence of polymorphonuclear leucocytes to an albumin-coated glass surface. Effects of therapeutic concentrations of the Catharantus derivatives vincristine, vinblastine and vindesine. Acta Med Scand 221:109-113.

Dai J, Ting-Beall HP, and Sheetz MP. 1997. The secretion-coupled endocytosis correlates with membrane tension changes in RBL 2H3 cells. J Gen Physiol 110:1-10.

Daleke DL. 2003. Regulation of transbilayer plasma membrane phospholipid asymmetry. J Lipid Res 44:233-242.

Danielsen EM and Hansen GH. 2006. Lipid raft organization and function in brush borders of epithelial cells. Mol Membr Biol 23:71-79.

Darlington PJ, Baroja ML, Chau TA, Siu E, Ling V, Carreno BM, and Madrenas J. 2002. Surface cytotoxic T lymphocyte-associated antigen 4 partitions within lipid rafts and relocates to the immunological synapse under conditions of inhibition of T cell activation. J Exp Med 195:1337-1347.

Davis BH, Walter RJ, Pearson CB, Becker EL, and Oliver JM. 1982. Membrane activity and topography of F-Met-Leu-Phe-Treated polymorphonuclear leukocytes. Acute and sustained responses to chemotactic peptide. Am J Pathol 108:206-216.

Davy A, Feuerstein C, and Robbins SM. 2000b. Signaling within a caveolae-like membrane microdomain in human neuroblastoma cells in response to fibroblast growth factor. J Neurochem 74:676-683.

Davy DA, Ball EE, Matthaei KI, Campbell HD, and Crouch MF. 2000a. The flightless I protein localizes to actin-based structures during embryonic development. Immunol Cell Biol 78:423-429.

DeBruin LS, Haines JD, Wellhauser LA, Radeva G, Schonmann V, Bienzle D, and Harauz G. 2005. Developmental partitioning of myelin basic protein into membrane microdomains. J Neurosci Res 80:211-225.

Dehghani ZA, Seveau S, Halbwachs-Mecarelli L, and Keller HU. 2003. Chemotactically-induced redistribution of CD43 as related to polarity and locomotion of human polymorphonuclear leucocytes. Biol Cell 95:265-273.

Dell'Angelica EC, Mullins C, Caplan S, and Bonifacino JS. 2000. Lysosome-related organelles. FASEB J 14:1265-1278.

Demaurex N, Monod A, Lew DP, and Krause KH. 1994. Characterization of receptor-mediated and store-regulated Ca2+ influx in human neutrophils. Biochem J 297 (Pt 3):595-601.

Denning DW, Venkateswarlu K, Oakley KL, Anderson MJ, Manning NJ, Stevens DA, Warnock DW, and Kelly SL. 1997. Itraconazole resistance in Aspergillus fumigatus. Antimicrob Agents Chemother 41:1364-1368.

Deretic D, Traverso V, Parkins N, Jackson F, Rodriguez de Turco EB, and Ransom N. 2004. Phosphoinositides, ezrin/moesin, and rac1 regulate fusion of rhodopsin transport carriers in retinal photoreceptors. Mol Biol Cell 15:359-370.

Deriy LV, Chor J, and Thomas LL. 2000. Surface expression of lactoferrin by resting neutrophils. Biochem Biophys Res Commun 275:241-246.

Dev KK, Nakanishi S, and Henley JM. 2001. Regulation of mglu(7) receptors by proteins that interact with the intracellular C-terminus. Trends Pharmacol Sci 22:355-361.

Devaux P and McConnell HM. 1972. Lateral diffusion in spin-labeled phosphatidylcholine multilayers. J Am Chem Soc 94:4475-4481.

Devaux PF. 1991. Static and dynamic lipid asymmetry in cell membranes. Biochemistry 30:1163-1173.

Diaz-Valencia JD, Almaraz-Barrera MJ, Arias-Romero LE, Hernandez-Rivas R, Rojo-Dominguez A, Guillen N, and Vargas M. 2005. The ABP-120 C-end region from Entamoeba histolytica interacts with sulfatide, a new lipid target. Biochem Biophys Res Commun 338:1527-1536.

Dienz O, Moller A, Strecker A, Stephan N, Krammer PH, Droge W, and Schmitz ML. 2003. Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa and

phospholipase C gamma 1 are required for NF-kappa B activation and lipid raft recruitment of protein kinase C theta induced by T cell costimulation. J Immunol 170:365-372.

Dietrich C, Bagatolli LA, Volovyk ZN, Thompson NL, Levi M, Jacobson K, and Gratton E. 2001. Lipid rafts reconstituted in model membranes. Biophys J 80:1417-1428.

Dietrich C, Merkel R, and Tampe R. 1997. Diffusion measurement of fluorescence-labeled amphiphilic molecules with a standard fluorescence microscope. Biophys J 72:1701-1710.

Ding J, Wu Z, Crider BP, Ma Y, Li X, Slaughter C, Gong L, and Xie XS. 2000. Identification and functional expression of four isoforms of ATPase II, the putative aminophospholipid translocase. Effect of isoform variation on the ATPase activity and phospholipid specificity. J Biol Chem 275:23378-23386.

DiNubile MJ, Cassimeris L, Joyce M, and Zigmond SH. 1995. Actin filament barbed-end capping activity in neutrophil lysates: the role of capping protein-beta 2. Mol Biol Cell 6:1659-1671.

DiNubile MJ and Huang S. 1997. High concentrations of phosphatidylinositol-4,5-bisphosphate may promote actin filament growth by three potential mechanisms: inhibiting capping by neutrophil lysates, severing actin filaments and removing capping protein-beta2 from barbed ends. Biochim Biophys Acta 1358:261-278.

Divecha N, Banfic H, and Irvine RF. 1991. The polyphosphoinositide cycle exists in the nuclei of Swiss 3T3 cells under the control of a receptor (for IGF-I) in the plasma membrane, and stimulation of the cycle increases nuclear diacylglycerol and apparently induces translocation of protein kinase C to the nucleus. EMBO J 10:3207-3214.

Djerdjouri B, Lenoir M, Giroud JP, and Perianin A. 1999. Contribution of mitogen-activated protein kinase to stimulation of phospholipase D by the chemotactic peptide fMet-Leu-Phe in human neutrophils. Biochem Biophys Res Commun 264:371-375.

Dobrowsky RT. 2000. Sphingolipid signalling domains floating on rafts or buried in caves? Cell Signal 12:81-90.

Domalewski MD, Guyer DA, Freer RJ, Muthukumaraswamy N, and Sklar LA. 1996. Fixation traps formyl peptide receptors in high and low affinity forms that can be regulated by GTP[S] in the absence of ligand. J Recept Signal Transduct Res 16:59-75.

Dong X, Mo Z, Bokoch G, Guo C, Li Z, and Wu D. 2005. P-Rex1 is a primary Rac2 guanine nucleotide exchange factor in mouse neutrophils. Curr Biol 15:1874-1879.

Doughman RL, Firestone AJ, and Anderson RA. 2003. Phosphatidylinositol phosphate kinases put PI4,5P(2) in its place. J Membr Biol 194:77-89.

Downes CP, Gray A, and Lucocq JM. 2005. Probing phosphoinositide functions in signaling and membrane trafficking. Trends Cell Biol 15:259-268.

Downey GP, Butler JR, Tapper H, Fialkow L, Saltiel AR, Rubin BB, and Grinstein S. 1998. Importance of MEK in neutrophil microbicidal responsiveness. J Immunol 160:434-443.

Duband JL, Nuckolls GH, Ishihara A, Hasegawa T, Yamada KM, Thiery JP, and Jacobson K. 1988. Fibronectin receptor exhibits high lateral mobility in embryonic locomoting cells but is immobile in focal contacts and fibrillar streaks in stationary cells. J Cell Biol 107:1385-1396.

Dupou L, Lopez A, and Tocanne JF. 1988. Comparative study of the lateral motion of extrinsic probes and anthracene-labelled constitutive phospholipids in the plasma membrane of Chinese hamster ovary cells. Eur J Biochem 171:669-674.

Durstin M, McColl SR, Gomez-Cambronero J, Naccache PH, and Sha'afi RI. 1993. Up-regulation of the amount of Gi alpha 2 associated with the plasma membrane in human neutrophils stimulated by granulocyte-macrophage colony-stimulating factor. Biochem J 292 (Pt 1):183-187.

Eddy RJ, Pierini LM, and Maxfield FR. 2002. Microtubule asymmetry during neutrophil polarization and migration. Mol Biol Cell 13:4470-4483.

Edidin M and Stroynowski I. 1991. Differences between the lateral organization of conventional and inositol phospholipid-anchored membrane proteins. A further definition of micrometer scale membrane domains. J Cell Biol 112:1143-1150.

Edidin M, Zuniga MC, and Sheetz MP. 1994. Truncation mutants define and locate cytoplasmic barriers to lateral mobility of membrane glycoproteins. Proc Natl Acad Sci U S A 91:3378-3382.

El Far O, Airas J, Wischmeyer E, Nehring RB, Karschin A, and Betz H. 2000. Interaction of the C-terminal tail region of the metabotropic glutamate receptor 7 with the protein kinase C substrate PICK1. Eur J Neurosci 12:4215-4221.

El Far O and Betz H. 2002. G-protein-coupled receptors for neurotransmitter amino acids: C-terminal tails, crowded signalosomes. Biochem J 365:329-336.

Eng RH, Bishburg E, Smith SM, Geller H, and Kapila R. 1986. Bacteremia and fungemia in patients with acquired immune deficiency syndrome. Am J Clin Pathol 86:105-107.

Enz R. 2002. The actin-binding protein Filamin-A interacts with the metabotropic glutamate receptor type 7. FEBS Lett 514:184-188.

Erickson RW, Langel-Peveri P, Traynor-Kaplan AE, Heyworth PG, and Curnutte JT. 1999. Activation of human neutrophil NADPH oxidase by phosphatidic acid or diacylglycerol in a cell-free system. Activity of diacylglycerol is dependent on its conversion to phosphatidic acid. J Biol Chem 274:22243-22250.

Exton JH. 1994. Phosphatidylcholine breakdown and signal transduction. Biochim Biophys Acta 1212:26-42.

Exton JH. 1997. New developments in phospholipase D. J Biol Chem 272:15579-15582.

Fabre S, Lang V, Harriague J, Jobart A, Unterman TG, Trautmann A, and Bismuth G. 2005. Stable activation of phosphatidylinositol 3-kinase in the T cell immunological synapse stimulates Akt signaling to FoxO1 nuclear exclusion and cell growth control. J Immunol 174:4161-4171.

Feder TJ, Brust-Mascher I, Slattery JP, Baird B, and Webb WW. 1996. Constrained diffusion or immobile fraction on cell surfaces: a new interpretation. Biophys J 70:2767-2773.

Fehr J. 1977. Complement as a mediator of granulocyte adherence and margination: studies based on the acute neutropenia of filtration leukapheresis. Prog Clin Biol Res 13:243-258.

Fehr J and Dahinden C. 1979. Modulating influence of chemotactic factor-induced cell adhesiveness on granulocyte function. J Clin Invest 64:8-16.

Fehr J and Jacob HS. 1977. In vitro granulocyte adherence and in vivo margination: two associated complement-dependent functions. Studies based on the acute neutropenia of filtration leukophoresis. J Exp Med 146:641-652.

Fensome A, Whatmore J, Morgan C, Jones D, and Cockcroft S. 1998. ADP-ribosylation factor and Rho proteins mediate fMLP-dependent activation of phospholipase D in human neutrophils. J Biol Chem 273:13157-13164.

Ferrell JE, Lee KJ, and Huestis WH. 1985. Membrane bilayer balance and erythrocyte shape: a quantitative assessment. Biochemistry 24:2849-2857.

Fessler MB, Arndt PG, Frasch SC, Lieber JG, Johnson CA, Murphy RC, Nick JA, Bratton DL, Malcolm KC, and Worthen GS. 2004. Lipid rafts regulate lipopolysaccharide-induced activation of Cdc42 and inflammatory functions of the human neutrophil. J Biol Chem 279:39989-39998.

Field CM and Kellogg D. 1999. Septins: cytoskeletal polymers or signalling GTPases? Trends Cell Biol 9:387-394.

Fielding CJ and Fielding PE. 2000. Cholesterol and caveolae: structural and functional relationships. Biochim Biophys Acta 1529:210-222.

Firtel RA and Chung CY. 2000. The molecular genetics of chemotaxis: sensing and responding to chemoattractant gradients. Bioessays 22:603-615.

Fra AM, Williamson E, Simons K, and Parton RG. 1994. Detergent-insoluble glycolipid microdomains in lymphocytes in the absence of caveolae. J Biol Chem 269:30745-30748.

Frasch SC, Henson PM, Nagaosa K, Fessler MB, Borregaard N, and Bratton DL. 2004. Phospholipid flip-flop and phospholipid scramblase 1 (PLSCR1) co-localize to uropod rafts in formylated Met-Leu-Phe-stimulated neutrophils. J Biol Chem 279:17625-17633.

Fredriksson M, Bergstrom K, and Asman B. 2002. IL-8 and TNF-alpha from peripheral neutrophils and acute-phase proteins in periodontitis. J Clin Periodontol 29:123-128.

Friedrichson T and Kurzchalia TV. 1998. Microdomains of GPI-anchored proteins in living cells revealed by crosslinking. Nature 394:802-805.

Frohman MA, Sung TC, and Morris AJ. 1999. Mammalian phospholipase D structure and regulation. Biochim Biophys Acta 1439:175-186.

Fujita H, Laham LE, Janmey PA, Kwiatkowski DJ, Stossel TP, Banno Y, Nozawa Y, Mullauer L, Ishizaki A, and Kuzumaki N. 1995. Functions of [His321]gelsolin isolated from a flat revertant of ras-transformed cells. Eur J Biochem 229:615-620.

Fujita T, Zawawi KH, Kurihara H, and Van Dyke TE. 2005. CD38 cleavage in. Cell Signal 17:167-175.

Funamoto S, Meili R, Lee S, Parry L, and Firtel RA. 2002. Spatial and temporal regulation of 3-phosphoinositides by PI 3-kinase and PTEN mediates chemotaxis. Cell 109:611-623.

Funatsu N, Kumanogoh H, Sokawa Y, and Maekawa S. 2000. Identification of gelsolin as an actin regulatory component in a triton insoluble low density fraction (raft) of newborn bovine brain. Neurosci Res 36:311-317.

Gaede HC and Gawrisch K. 2003. Lateral diffusion rates of lipid, water, and a hydrophobic drug in a multilamellar liposome. Biophys J 85:1734-1740.

Galandrini R, Tassi I, Mattia G, Lenti L, Piccoli M, Frati L, and Santoni A. 2002. SH2-containing inositol phosphatase (SHIP-1) transiently translocates to raft domains and modulates CD16-mediated cytotoxicity in human NK cells. Blood 100:4581-4589.

Gao JL, Becker EL, Freer RJ, Muthukumaraswamy N, and Murphy PM. 1994. A high potency nonformylated peptide agonist for the phagocyte N-formylpeptide chemotactic receptor. J Exp Med 180:2191-2197.

Gardiner EM, Pestonjamasp KN, Bohl BP, Chamberlain C, Hahn KM, and Bokoch GM. 2002. Spatial and temporal analysis of Rac activation during live neutrophil chemotaxis. Curr Biol 12:2029-2034.

Garland LG. 1992. New pathways of phagocyte activation: the coupling of receptor-linked phospholipase D and the role of tyrosine kinase in primed neutrophils. FEMS Microbiol Immunol 5:229-237.

Garnotel R, Monboisse JC, Randoux A, Haye B, and Borel JP. 1995. The binding of type I collagen to lymphocyte function-associated antigen (LFA) 1 integrin triggers the respiratory burst of human polymorphonuclear neutrophils. Role of calcium signaling and tyrosine phosphorylation of LFA 1. J Biol Chem 270:27495-27503.

Gascard P, Sauvage M, Sulpice JC, and Giraud F. 1993. Characterization of structural and functional phosphoinositide domains in human erythrocyte membranes. Biochemistry 32:5941-5948.

Gaudry M, Perianin A, Marquetty C, and Hakim J. 1988. Negative effect of a protein kinase C inhibitor (H-7) on human polymorphonuclear neutrophil locomotion. Immunology 63:715-719.

Gaus K, Rodriguez M, Ruberu KR, Gelissen I, Sloane TM, Kritharides L, and Jessup W. 2005. Domain-specific lipid distribution in macrophage plasma membranes. J Lipid Res 46:1526-1538.

Geiger B and Bershadsky A. 2001. Assembly and mechanosensory function of focal contacts. Curr Opin Cell Biol 13:584-592.

Gelas P, Von T, V, Record M, Baggiolini M, and Chap H. 1992. Human neutrophil phospholipase D activation by N-formylmethionyl-leucylphenylalanine reveals a two-step process for the control of phosphatidylcholine breakdown and oxidative burst. Biochem J 287 (Pt 1):67-72.

Gerisch G and Keller HU. 1981. Chemotactic reorientation of granulocytes stimulated with micropipettes containing fMet-Leu-Phe. J Cell Sci 52:1-10.

Gewirtz AT and Simons ER. 1997. Phospholipase D mediates Fc gamma receptor activation of neutrophils and provides specificity between high-valency immune complexes and fMLP signaling pathways. J Leukoc Biol 61:522-528.

Gilbert TL, Bennett TA, Maestas DC, Cimino DF, and Prossnitz ER. 2001. Internalization of the human N-formyl peptide and C5a chemoattractant receptors occurs via clathrin-independent mechanisms. Biochemistry 40:3467-3475.

Gilmore AP and Burridge K. 1996. Regulation of vinculin binding to talin and actin by phosphatidyl-inositol-4-5-bisphosphate. Nature 381:531-535.

Ginis I and Tauber AI. 1990. Activation mechanisms of adherent human neutrophils. Blood 76:1233-1239.

Godin DV and Garnett M. 1976. Perturbational effects of inorganic cations on human erythrocyte membranes. J Membr Biol 28:143-168.

Goldmann WH, Teodoridis JM, Sharma CP, Alonso JL, and Isenberg G. 1999. Fragments from alpha-actinin insert into reconstituted lipid bilayers. Biochem Biophys Res Commun 264:225-229.

Golub T and Caroni P. 2005. PI(4,5)P2-dependent microdomain assemblies capture microtubules to promote and control leading edge motility. J Cell Biol 169:151-165.

Golub T and Pico C. 2005. Spatial control of actin-based motility through plasmalemmal PtdIns(4,5)P2-rich raft assemblies. Biochem Soc Symp119-127.

Golub T, Wacha S, and Caroni P. 2004. Spatial and temporal control of signaling through lipid rafts. Curr Opin Neurobiol 14:542-550.

Gombos I, Bacso Z, Detre C, Nagy H, Goda K, Andrasfalvy M, Szabo G, and Matko J. 2004. Cholesterol sensitivity of detergent resistance: a rapid flow cytometric test for detecting constitutive or induced raft association of membrane proteins. Cytometry A 61:117-126.

Gomez-Mouton C, Abad JL, Mira E, Lacalle RA, Gallardo E, Jimenez-Baranda S, Illa I, Bernad A, Manes S, and Martinez A. 2001. Segregation of leading-edge and uropod components into specific lipid rafts during T cell polarization. Proc Natl Acad Sci U S A 98:9642-9647.

Goodlad GA and Clark CM. 1993. Actin and actin-binding proteins in plasma membranes derived from Walker 256 ascites or solid tumour cells. Biochim Biophys Acta 1145:177-179.

Haag-Weber M and Horl WH. 1995. The immune system in uremia and during its treatment. New Horiz 3:669-679.

Hakomori S. 2003. Structure, organization, and function of glycosphingolipids in membrane. Curr Opin Hematol 10:16-24.

Hakomori S. 2004. Carbohydrate-to-carbohydrate interaction, through glycosynapse, as a basis of cell recognition and membrane organization. Glycoconj J 21:125-137.

Hakomori S and Handa K. 2003. Interaction of glycosphingolipids with signal transducers and membrane proteins in glycosphingolipid-enriched microdomains. Methods Enzymol 363:191-207.

Hakomori S, Handa K, Iwabuchi K, Yamamura S, and Prinetti A. 1998a. New insights in glycosphingolipid function: "glycosignaling domain," a cell surface assembly of glycosphingolipids with signal transducer molecules,involved in cell adhesion coupled with signaling. Glycobiology 8:xi-xix.

Hakomori S, Yamamura S, and Handa AK. 1998b. Signal transduction through glyco(sphingo)lipids. Introduction and recent studies on glyco(sphingo)lipid-enriched microdomains. Ann N Y Acad Sci 845:1-10.

Hall RA. 2004. beta-Adrenergic receptors and their interacting proteins. Semin Cell Dev Biol 15:281-288.

Hall RA, Premont RT, and Lefkowitz RJ. 1999. Heptahelical receptor signaling: beyond the G protein paradigm. J Cell Biol 145:927-932.

Hanahan DJ and Chaikoff IL. 1947. A new phospholipid splitting enzyme specific for an ester linkage between the nitrogenous base and the phosphoric acid group. J Biol Chem 169:699-703.

Hanahan DJ and Chaikoff IL. 1948. On the nature of the phosphorus-containing lipides of cabbage leaves and their relation to a phospholipide-splitting enzyme contained in these leaves. J Biol Chem 172:191-198.

Handa K, Kojima N, and Hakomori S. 2000. Analysis of glycolipid-dependent cell adhesion based on carbohydrate-carbohydrate interaction. Methods Enzymol 312:447-458.

Hansen GH, Niels-Christiansen LL, Immerdal L, Hunziker W, Kenny AJ, and Danielsen EM. 1999. Transcytosis of immunoglobulin A in the mouse enterocyte occurs through glycolipid raft- and rab17-containing compartments. Gastroenterology 116:610-622.

Hansen GH, Niels-Christiansen LL, Thorsen E, Immerdal L, and Danielsen EM. 2000. Cholesterol depletion of enterocytes. Effect on the Golgi complex and apical membrane trafficking. J Biol Chem 275:5136-5142.

Harder T and Engelhardt KR. 2004. Membrane domains in lymphocy. Traffic 5:265-275.

Harder T, Scheiffele P, Verkade P, and Simons K. 1998. Lipid domain structure of the plasma membrane revealed by patching of membrane components. J Cell Biol 141:929-942.

Haribabu B, Richardson RM, Verghese MW, Barr AJ, Zhelev DV, and Snyderman R. 2000. Function and regulation of chemoattractant receptors. Immunol Res 22:271-279.

Harlan JE, Hajduk PJ, Yoon HS, and Fesik SW. 1994. Pleckstrin homology domains bind to phosphatidylinositol-4,5-bisphosphate. Nature 371:168-170.

Harms GS, Sonnleitner M, Schutz GJ, Gruber HJ, and Schmidt T. 1999. Single-molecule anisotropy imaging. Biophys J 77:2864-2870.

Haroldsen PE, Clay KL, and Murphy RC. 1987. Quantitation of lyso-platelet activating factor molecular species from human neutrophils by mass spectrometry. J Lipid Res 28:42-49.

Harris TJ, Ravandi A, and Siu CH. 2001. Assembly of glycoprotein-80 adhesion complexes in Dictyostelium. Receptor compartmentalization and oligomerization in membrane rafts. J Biol Chem 276:48764-48774.

Harvath L, McCall CE, Bass DA, and McPhail LC. 1987. Inhibition of human neutrophil chemotaxis by the protein kinase inhibitor, 1-(5-isoquinolinesulfonyl) piperazine. J Immunol 139:3055-3061.

Hashida M, Fukushima A, Zhang J, Kodama H, and Ueno H. 2000. Involvement of superoxide generated by polymorphonuclear leukocytes in endotoxin-induced uveitis. Graefes Arch Clin Exp Ophthalmol 238:359-365.

Hashizume T, Nakao M, and Sato T. 1996. Sphingosine enhances phosphatidylinositol 4-kinase activity in rabbit platelets. J Biochem (Tokyo) 120:61-65.

Hattar K, Grandel U, Moeller A, Fink L, Iglhaut J, Hartung T, Morath S, Seeger W, Grimminger F, and Sibelius U. 2006. Lipoteichoic acid (LTA) from Staphylococcus aureus stimulates human neutrophil cytokine release by a CD14-dependent, Toll-like-receptor-independent mechanism: Autocrine role of tumor necrosis factor-[alpha] in mediating LTA-induced interleukin-8 generation. Crit Care Med 34:835-841.

Hattula K, Furuhjelm J, Arffman A, and Peranen J. 2002. A Rab8-specific GDP/GTP exchange factor is involved in actin remodeling and polarized membrane transport. Mol Biol Cell 13:3268-3280.

Hattula K and Peranen J. 2005. Purification and functional properties of a Rab8-specific GEF (Rabin3) in action remodeling and polarized transport. Methods Enzymol 403:284-295.

Haucke V. 2005. Phosphoinositide regulation of clathrin-mediated endocytosis. Biochem Soc Trans 33:1285-1289.

Haus U, Hartmann H, Trommler P, Noegel AA, and Schleicher M. 1991. F-actin capping by cap32/34 requires heterodimeric conformation and can be inhibited with PIP2. Biochem Biophys Res Commun 181:833-839.

He HT, Lellouch A, and Marguet D. 2005. Lipid rafts and the initiation of T cell receptor signaling. Semin Immunol 17:23-33.

Heiss SG and Cooper JA. 1991. Regulation of CapZ, an actin capping protein of chicken muscle, by anionic phospholipids. Biochemistry 30:8753-8758.

Helms JB and Zurzolo C. 2004. Lipids as targeting signals: lipid rafts and intracellular trafficking. Traffic 5:247-254.

Hemker HC, van Rijn JL, Rosing J, van Dieijen G, Bevers EM, and Zwaal RF. 1983. Platelet membrane involvement in blood coagulation. Blood Cells 9:303-317.

Hermida D, Elezgarai I, Puente N, Alonso V, Anabitarte N, Bilbao A, Donate-Oliver F, and Grandes P. 2006. Developmental increase in postsynaptic alpha-amino-3-hydroxy-5-methyl-4 isoxazolepropionic acid receptor compartmentalization at the calyx of held synapse. J Comp Neurol 495:624-634.

Hii CS, Stacey K, Moghaddami N, Murray AW, and Ferrante A. 1999. Role of the extracellular signal-regulated protein kinase cascade in human neutrophil killing of Staphylococcus aureus and Candida albicans and in migration. Infect Immun 67:1297-1302.

Hiltbold EM, Poloso NJ, and Roche PA. 2003. MHC class II-peptide complexes and APC lipid rafts accumulate at the immunological synapse. J Immunol 170:1329-1338.

Hjalm G, MacLeod RJ, Kifor O, Chattopadhyay N, and Brown EM. 2001. Filamin-A binds to the carboxyl-terminal tail of the calcium-sensing receptor, an interaction that participates in CaR-mediated activation of mitogen-activated protein kinase. J Biol Chem 276:34880-34887.

Hodgkin MN, Masson MR, Powner D, Saqib KM, Ponting CP, and Wakelam MJ. 2000. Phospholipase D regulation and localisation is dependent upon a phosphatidylinositol 4,5-biphosphate-specific PH domain. Curr Biol 10:43-46.

Hoer A, Cetindag C, and Oberdisse E. 2000. Influence of phosphatidylinositol 4,5-bisphosphate on human phospholipase D1 wild-type and deletion mutants: is there evidence for an interaction of phosphatidylinositol 4,5-bisphosphate with the putative pleckstrin homology domain? Biochim Biophys Acta 1481:189-201.

Hofer D, Jons T, Kraemer J, and Drenckhahn D. 1998. From cytoskeleton to polarity and chemoreception in the gut epithelium. Ann N Y Acad Sci 859:75-84.

Hollan S. 1996. Membrane fluidity of blood cells. Haematologia (Budap) 27:109-127.

Holopainen JM, Angelova MI, and Kinnunen PK. 2000. Vectorial budding of vesicles by asymmetrical enzymatic formation of ceramide in giant liposomes. Biophys J 78:830-838.

Holowka D and Baird B. 2001. Fc(epsilon)RI as a paradigm for a lipid raft-dependent receptor in hematopoietic cells. Semin Immunol 13:99-105.

Holowka D, Sheets ED, and Baird B. 2000. Interactions between Fc(epsilon)RI and lipid raft components are regulated by the actin cytoskeleton. J Cell Sci 113:2-19.

Honeycutt PJ and Niedel JE. 1986. Cytochalasin B enhancement of the diacylglycerol response in formyl peptide-stimulated neutrophils. J Biol Chem 261:15900-15905.

Hooper NM. 1999. Detergent-insoluble glycosphingolipid/cholesterol-rich membrane domains, lipid rafts and caveolae (review). Mol Membr Biol 16:145-156.

Hopkins CR, Gibson A, Shipman M, Strickland DK, and Trowbridge IS. 1994. In migrating fibroblasts, recycling receptors are concentrated in narrow tubules in the pericentriolar area, and then routed to the plasma membrane of the leading lamella. J Cell Biol 125:1265-1274.

Horl WH. 2002. Hemodialysis membranes: interleukins, biocompatibility, and middle molecules. J Am Soc Nephrol 13 Suppl 1:S62-S71.

Huang S, Lifshitz L, Patki-Kamath V, Tuft R, Fogarty K, and Czech MP. 2004. Phosphatidylinositol-4,5-bisphosphate-rich plasma membrane patches organize active zones of endocytosis and ruffling in cultured adipocytes. Mol Cell Biol 24:9102-9123.

Hurley JH and Misra S. 2000. Signaling and subcellular targeting by membrane-binding domains. Annu Rev Biophys Biomol Struct 29:49-79.

Ikonen E and Simons K. 1998. Protein and lipid sorting from the trans-Golgi network to the plasma membrane in polarized cells. Semin Cell Dev Biol 9:503-509.

Ilangumaran S, Borisch B, and Hoessli DC. 1999. Signal transduction via CD44: role of plasma membrane microdomains. Leuk Lymphoma 35:455-469.

Innocenti M, Zucconi A, Disanza A, Frittoli E, Areces LB, Steffen A, Stradal TE, Di Fiore PP, Carlier MF, and Scita G. 2004. Abi1 is essential for the formation and activation of a WAVE2 signalling complex. Nat Cell Biol 6:319-327.

Ito T, Williams JD, Fraser DJ, and Phillips AO. 2004. Hyaluronan regulates transforming growth factor-beta1 receptor compartmentalization. J Biol Chem 279:25326-25332.

Iwabuchi K, Handa K, and Hakomori S. 2000. Separation of glycosphingolipid-enriched microdomains from caveolar membrane characterized by presence of caveolin. Methods Enzymol 312:488-494.

Iwabuchi K, Yamamura S, Prinetti A, Handa K, and Hakomori S. 1998. GM3-enriched microdomain involved in cell adhesion and signal transduction through carbohydrate-carbohydrate interaction in mouse melanoma B16 cells. J Biol Chem 273:9130-9138.

Iwata M, Edashige K, and Saeki K. 1990. Activation of protein kinase C by myristate and its requirements of Ca2+ and phospholipid. Physiol Chem Phys Med NMR 22:211-218.

Iyer SS, Barton JA, Bourgoin S, and Kusner DJ. 2004. Phospholipases D1 and D2 coordinately regulate macrophage phagocytosis. J Immunol 173:2615-2623.

Jacobson K and Dietrich C. 1999. Looking at lipid rafts? Trends Cell Biol 9:87-91.

Jahn R, Lang T, and Sudhof TC. 2003. Membrane fusion. Cell 112:519-533.

Jahn R and Sudhof TC. 1999. Membrane fusion and exocytosis. Annu Rev Biochem 68:863-911.

Janes PW, Ley SC, and Magee AI. 1999. Aggregation of lipid rafts accompanies signaling via the T cell antigen receptor. J Cell Biol 147:447-461.

Janmey PA. 1994. Phosphoinositides and calcium as regulators of cellular actin assembly and disassembly. Annu Rev Physiol 56:169-191.

Janmey PA and Stossel TP. 1989. Gelsolin-polyphosphoinositide interaction. Full expression of gelsolin-inhibiting function by polyphosphoinositides in vesicular form and inactivation by dilution, aggregation, or masking of the inositol head group. J Biol Chem 264:4825-4831.

Janmey PA, Xian W, and Flanagan LA. 1999. Controlling cytoskeleton structure by phosphoinositide-protein interactions: phosphoinositide binding protein domains and effects of lipid packing. Chem Phys Lipids 101:93-107.

Jesaitis AJ, Bokoch GM, Tolley JO, and Allen RA. 1988. Lateral segregation of neutrophil chemotactic receptors into actin- and fodrin-rich plasma membrane microdomains depleted in guanyl nucleotide regulatory proteins. J Cell Biol 107:921-928.

Jesaitis AJ, Erickson RW, Klotz KN, Bommakanti RK, and Siemsen DW. 1993. Functional molecular complexes of human N-formyl chemoattractant receptors and actin. J Immunol 151:5653-5665.

Jesaitis AJ and Klotz KN. 1993. Cytoskeletal regulation of chemotactic receptors: molecular complexation of N-formyl peptide receptors with G proteins and actin. Eur J Haematol 51:288-293.

Jesaitis AJ, Naemura JR, Sklar LA, Cochrane CG, and Painter RG. 1984. Rapid modulation of N-formyl chemotactic peptide receptors on the surface of human granulocytes: formation of high-affinity ligand-receptor complexes in transient association with cytoskeleton. J Cell Biol 98:1378-1387.

Jesaitis AJ, Tolley JO, and Allen RA. 1986. Receptor-cytoskeleton interactions and membrane traffic may regulate chemoattractant-induced superoxide production in human granulocytes. J Biol Chem 261:13662-13669.

Jesaitis AJ, Tolley JO, Bokoch GM, and Allen RA. 1989. Regulation of chemoattractant receptor interaction with transducing proteins by organizational control in the plasma membrane of human neutrophils. J Cell Biol 109:2783-2790.

Jesaitis AJ, Tolley JO, Painter RG, Sklar LA, and Cochrane CG. 1985. Membrane-cytoskeleton interactions and the regulation of chemotactic peptide-induced activation of human granulocytes: the effects of dihydrocytochalasin B. J Cell Biochem 27:241-253.

Jiang X, Kobayashi T, Nahirney PC, Garcia dS, and Seguchi H. 1996. Ultracytochemical localization of alkaline phosphatase (ALPase) activity in neutrophils of the rat lung following injection of lipopolysaccharide. Kaibogaku Zasshi 71:183-194.

Jiang X, Kobayashi T, Nahirney PC, Garcia dS, and Seguchi H. 1998. An ultracytochemical study on the dynamics of alkaline phosphatase-positive granules in rat neutrophils. Histol Histopathol 13:57-65.

Jiao X, Zhang N, Xu X, Oppenheim JJ, and Jin T. 2005. Ligand-induced partitioning of human CXCR1 chemokine receptors with lipid raft microenvironments facilitates G-protein-dependent signaling. Mol Cell Biol 25:5752-5762.

Jin T, Zhang N, Long Y, Parent CA, and Devreotes PN. 2000. Localization of the G protein betagamma complex in living cells during chemotaxis. Science 287:1034-1036.

Johansson A, Jesaitis AJ, Lundqvist H, Magnusson KE, Sjolin C, Karlsson A, and Dahlgren C. 1995. Different subcellular localization of cytochrome b and the dormant NADPH-oxidase in neutrophils and macrophages: effect on the production of reactive oxygen species during phagocytosis. Cell Immunol 161:61-71.

Johansson B, Wymann MP, Holmgren-Peterson K, and Magnusson KE. 1993. N-formyl peptide receptors in human neutrophils display distinct membrane distribution and lateral mobility when labeled with agonist and antagonist. J Cell Biol 121:1281-1289.

Jones SL and Brown EJ. 1996. FcgammaRII-mediated adhesion and phagocytosis induce L-plastin phosphorylation in human neutrophils. J Biol Chem 271:14623-14630.

Jordan BA, Trapaidze N, Gomes I, Nivarthi R, and Devi LA. 2001. Oligomerization of opioid receptors with beta 2-adrenergic receptors: a role in trafficking and mitogen-activated protein kinase activation. Proc Natl Acad Sci U S A 98:343-348.

Jost M, Simpson F, Kavran JM, Lemmon MA, and Schmid SL. 1998. Phosphatidylinositol-4,5-bisphosphate is required for endocytic coated vesicle formation. Curr Biol 8:1399-1402.

Jozsef L, Khreiss T, El Kebir D, and Filep JG. 2006. Activation of TLR-9 induces IL-8 secretion through peroxynitrite signaling in human neutrophils. J Immunol 176:1195-1202.

Jugloff LS and Jongstra-Bilen J. 1997. Cross-linking of the IgM receptor induces rapid translocation of IgM-associated Ig alpha, Lyn, and Syk tyrosine kinases to the membrane skeleton. J Immunol 159:1096-1106.

Kaldi K, Szeberenyi J, Rada BK, Kovacs P, Geiszt M, Mocsai A, and Ligeti E. 2002. Contribution of phopholipase D and a brefeldin A-sensitive ARF to chemoattractant-induced superoxide production and secretion of human neutrophils. J Leukoc Biol 71:695-700.

Kanaho Y, Kanoh H, Saitoh K, and Nozawa Y. 1991. Phospholipase D activation by platelet-activating factor, leukotriene B4, and formyl-methionyl-leucyl-phenylalanine in rabbit neutrophils. Phospholipase D activation is involved in enzyme release. J Immunol 146:3536-3541.

Kaplan MR and Simoni RD. 1985. Transport of cholesterol from the endoplasmic reticulum to the plasma membrane. J Cell Biol 101:446-453.

Karnell FG, Brezski RJ, King LB, Silverman MA, and Monroe JG. 2005. Membrane cholesterol content accounts for developmental differences in surface B cell receptor compartmentalization and signaling. J Biol Chem 280:25621-25628.

Kasahara K and Sanai Y. 1999. Possible roles of glycosphingolipids in lipid rafts. Biophys Chem 82:121-127.

Katanaev VL. 2001. Signal transduction in neutrophil chemotaxis. Biochemistry (Mosc) 66:351-368.

Keil ML, Solomon NL, Lodhi IJ, Stone KC, Jesaitis AJ, Chang PS, Linderman JJ, and Omann GM. 2003. Priming-induced localization of G(ialpha2) in high density membrane microdomains. Biochem Biophys Res Commun 301:862-872.

Kenworthy AK and Edidin M. 1998. Distribution of a glycosylphosphatidylinositol-anchored protein at the apical surface of MDCK cells examined at a resolution of <100 A using imaging fluorescence resonance energy transfer. J Cell Biol 142:69-84.

Kessels GC, Krause KH, and Verhoeven AJ. 1993. Protein kinase C activity is not involved in N-formylmethionyl-leucyl-phenylalanine-induced phospholipase D activation in human neutrophils, but is essential for concomitant NADPH oxidase activation: studies with a staurosporine analogue with improved selectivity for protein kinase C. Biochem J 292 (Pt 3):781-785.

Kessels GC, Roos D, and Verhoeven AJ. 1991. fMet-Leu-Phe-induced activation of phospholipase D in human neutrophils. Dependence on changes in cytosolic free Ca2+ concentration and relation with respiratory burst activation. J Biol Chem 266:23152-23156.

Kettritz R, Jennette JC, and Falk RJ. 1997. Crosslinking of ANCA-antigens stimulates superoxide release by human neutrophils. J Am Soc Nephrol 8:386-394.

Key TA, Bennett TA, Foutz TD, Gurevich VV, Sklar LA, and Prossnitz ER. 2001. Regulation of formyl peptide receptor agonist affinity by reconstitution with arrestins and heterotrimeric G proteins. J Biol Chem 276:49204-49212.

Key TA, Foutz TD, Gurevich VV, Sklar LA, and Prossnitz ER. 2003. N-formyl peptide receptor phosphorylation domains differentially regulate arrestin and agonist affinity. J Biol Chem 278:4041-4047.

Kiehn TE and Armstrong D. 1990. Changes in the spectrum of organisms causing bacteremia and fungemia in immunocompromised patients due to venous access devices. Eur J Clin Microbiol Infect Dis 9:869-872.

Kirkpatrick BL, Glover SC, Reeves DS, and MacGowan AP. 1997. Microbiologically proven bacterial infections in AIDS. Postgrad Med J 73:565-570.

Kjeldsen L, Bainton DF, Sengelov H, and Borregaard N. 1994. Identification of neutrophil gelatinase-associated lipocalin as a novel matrix protein of specific granules in human neutrophils. Blood 83:799-807.

Klein JB, Scherzer JA, Harding G, Jacobs AA, and McLeish KR. 1995. TNF-alpha stimulates increased plasma membrane guanine nucleotide binding protein activity in polymorphonuclear leukocytes. J Leukoc Biol 57:500-506.

Klotz KN and Jesaitis AJ. 1994a. Neutrophil chemoattractant receptors and the membrane skeleton. Bioessays 16:193-198.

Klotz KN and Jesaitis AJ. 1994b. The interaction of N-formyl peptide chemoattractant receptors with the membrane skeleton is energy-dependent. Cell Signal 6:943-947.

Klotz KN, Krotec KL, Gripentrog J, and Jesaitis AJ. 1994. Regulatory interaction of N-formyl peptide chemoattractant receptors with the membrane skeleton in human neutrophils. J Immunol 152:801-810.

Knall C, Worthen GS, and Johnson GL. 1997. Interleukin 8-stimulated phosphatidylinositol-3-kinase activity regulates the migration of human neutrophils independent of extracellular signal-regulated kinase and p38 mitogen-activated protein kinases. Proc Natl Acad Sci U S A 94:3052-3057.

Kobayashi T and Robinson JM. 1991. A novel intracellular compartment with unusual secretory properties in human neutrophils. J Cell Biol 113:743-756.

Kobayashi T, Robinson JM, and Seguchi H. 1998a. Identification of intracellular sites of superoxide production in stimulated neutrophils. J Cell Sci 111 (Pt 1):81-91.

Kobayashi T, Zinchuk VS, Garcia dS, Jiang F, Yamasaki Y, Kataoka S, Okada T, Tsunawaki S, and Seguchi H. 2000. Suppressive effect of rebamipide, an antiulcer agent, against activation of human neutrophils exposed to formyl-methionyl-leucyl-phenylalanine. Histol Histopathol 15:1067-1076.

Kobayashi T, Zinchuk VS, Okada T, del Saz EG, and Seguchi H. 1998b. Intracellular dynamics of alkaline phosphatase-containing granules in electropermeabilized human neutrophils. Histochem Cell Biol 110:395-406.

Kocher M, Edberg JC, Fleit HB, and Kimberly RP. 1998. Antineutrophil cytoplasmic antibodies preferentially engage Fc gammaRIIIb on human neutrophils. J Immunol 161:6909-6914.

Koenderman L, Yazdanbakhsh M, Roos D, and Verhoeven AJ. 1989. Dual mechanisms in priming of the chemoattractant-induced respiratory burst in human granulocytes. A Ca2+-dependent and a Ca2+-independent route. J Immunol 142:623-628.

Kojima N and Hakomori S. 1991. Cell adhesion, spreading, and motility of GM3-expressing cells based on glycolipid-glycolipid interaction. J Biol Chem 266:17552-17558.

Kojima N, Shiota M, Sadahira Y, Handa K, and Hakomori S. 1992. Cell adhesion in a dynamic flow system as compared to static system. Glycosphingolipid-glycosphingolipid interaction in the dynamic system predominates over le. J Biol Chem 267:17264-17270.

Koppel DE, Sheetz MP, and Schindler M. 1981. Matrix control of protein diffusion in biological membranes. Proc Natl Acad Sci U S A 78:3576-3580.

Kornberg RD and McConnell HM. 1971. Inside-outside transitions of phospholipids in vesicle membranes. Biochemistry 10:1111-1120.

Kovarova M, Tolar P, Arudchandran R, Draberova L, Rivera J, and Draber P. 2001. Structure-function analysis of Lyn kinase association with lipid rafts and initiation of early signaling events after Fcepsilon receptor I aggregation. Mol Cell Biol 21:8318-8328.

Kramer EM, Klein C, Koch T, Boytinck M, and Trotter J. 1999. Compartmentation of Fyn kinase with glycosylphosphatidylinositol-anchored molecules in oligodendrocytes facilitates kinase activation during myelination. J Biol Chem 274:29042-29049.

Kreienkamp HJ. 2002. Organisation of G-protein-coupled receptor signalling complexes by scaffolding proteins. Curr Opin Pharmacol 2:581-586.

Krumholz HM, Sande MA, and Lo B. 1989. Community-acquired bacteremia in patients with acquired immunodeficiency syndrome: clinical presentation, bacteriology, and outcome. Am J Med 86:776-779.

Ktistakis NT, Brown HA, Sternweis PC, and Roth MG. 1995. Phospholipase D is present on Golgi-enriched membranes and its activation by ADP ribosylation factor is sensitive to brefeldin A. Proc Natl Acad Sci U S A 92:4952-4956.

Ktistakis NT, Brown HA, Waters MG, Sternweis PC, and Roth MG. 1996. Evidence that phospholipase D mediates ADP ribosylation factor-dependent formation of Golgi coated vesicles. J Cell Biol 134:295-306.

Kumashi P, Girgawy E, Tarrand JJ, Rolston KV, Raad II, and Safdar A. 2005. Streptococcus pneumoniae bacteremia in patients with cancer: disease characteristics and outcomes in the era of escalating drug resistance (1998-2002). Medicine (Baltimore) 84:303-312.

Kundu A, Avalos RT, Sanderson CM, and Nayak DP. 1996. Transmembrane domain of influenza virus neuraminidase, a type II protein, possesses an apical sorting signal in polarized MDCK cells. J Virol 70:6508-6515.

Kurzchalia TV and Parton RG. 1999. Membrane microdomains and caveolae. Curr Opin Cell Biol 11:424-431.

Kusumi A, Ike H, Nakada C, Murase K, and Fujiwara T. 2005. Single-molecule tracking of membrane molecules: plasma membrane compartmentalization and dynamic assembly of raft-philic signaling molecules. Semin Immunol 17:3-21.

Kwiatkowska K, Frey J, and Sobota A. 2003. Phosphorylation of FcgammaRIIA is required for the receptor-induced actin rearrangement and capping: the role of membrane rafts. J Cell Sci 116:537-550.

Ladha S, Mackie AR, Harvey LJ, Clark DC, Lea EJ, Brullemans M, and Duclohier H. 1996. Lateral diffusion in planar lipid bilayers: a fluorescence recovery after

photobleaching investigation of its modulation by lipid composition, cholesterol, or alamethicin content and divalent cations. Biophys J 71:1364-1373.

Lafont F and Simons K. 2001. Raft-partitioning of the ubiquitin ligases Cbl and Nedd4 upon IgE-triggered cell signaling. Proc Natl Acad Sci U S A 98:3180-3184.

Lafont F, Verkade P, Galli T, Wimmer C, Louvard D, and Simons K. 1999. Raft association of SNAP receptors acting in apical trafficking in Madin-Darby canine kidney cells. Proc Natl Acad Sci U S A 96:3734-3738.

Lassing I and Lindberg U. 1985. Specific interaction between phosphatidylinositol 4,5-bisphosphate and profilactin. Nature 314:472-474.

Lassing I and Lindberg U. 1988a. Evidence that the phosphatidylinositol cycle is linked to cell motility. Exp Cell Res 174:1-15.

Lassing I and Lindberg U. 1988b. Specificity of the interaction between phosphatidylinositol 4,5-bisphosphate and the profilin:actin complex. J Cell Biochem 37:255-267.

Lavastre V, Roberge CJ, Pelletier M, Gauthier M, and Girard D. 2002. Toxaphene, but Not Beryllium, Induces Human Neutrophil Chemotaxis and Apoptosis via Reactive Oxygen Species (ROS): Involvement of Caspases and ROS in the Degradation of Cytoskeletal Proteins. Clin Immunol 104:40-48.

Lebrand C, Corti M, Goodson H, Cosson P, Cavalli V, Mayran N, Faure J, and Gruenberg J. 2002. Late endosome motility depends on lipids via the small GTPase Rab7. EMBO J 21:1289-1300.

Lechler T, Shevchenko A, and Li R. 2000. Direct involvement of yeast type I myosins in Cdc42-dependent actin polymerization. J Cell Biol 148:363-373.

Lee AG. 1977. Lipid phase transitions and phase diagrams. I. Lipid phase transitions. Biochim Biophys Acta 472:237-281.

Lee HY, Kang HK, Jo EJ, Kim JI, Lee YN, Lee SH, Park YM, Ryu SH, Kwak JY, and Bae YS. 2004. Trp-Lys-Tyr-Met-Val-Met stimulates phagocytosis via phospho-lipase D-dependent signaling in mouse dendritic cells. Exp Mol Med 36:135-144.

Lee JS, Kim JH, Jang IH, Kim HS, Han JM, Kazlauskas A, Yagisawa H, Suh PG, and Ryu SH. 2005. Phosphatidylinositol (3,4,5)-trisphosphate specifically interacts with the phox homology domain of phospholipase D1 and stimulates its activity. J Cell Sci 118:4405-4413.

Lennartz MR. 1999. Phospholipases and phagocytosis: the role of phospholipid-derived second messengers in phagocytosis. Int J Biochem Cell Biol 31:415-430.

Levitz SM and Farrell TP. 1990. Human neutrophil degranulation stimulated by Aspergillus fumigatus. J Leukoc Biol 47:170-175.

Li S, Okamoto T, Chun M, Sargiacomo M, Casanova JE, Hansen SH, Nishimoto I, and Lisanti MP. 1995. Evidence for a regulated interaction between heterotrimeric G proteins and caveolin. J Biol Chem 270:15693-15701.

Li XA, Everson WV, and Smart EJ. 2005. Caveolae, lipid rafts, and vascular disease. Trends Cardiovasc Med 15:92-96.

Li Z, Hannigan M, Mo Z, Liu B, Lu W, Wu Y, Smrcka AV, Wu G, Li L, Liu M, Huang CK, and Wu D. 2003. Directional sensing requires G beta gamma-mediated PAK1 and PIX alpha-dependent activation of Cdc42. Cell 114:215-227.

Ligeti E and Mocsai A. 1999. Exocytosis of neutrophil granulocytes. Biochem Pharmacol 57:1209-1214.

Lin J and Shaw AS. 2005. Getting downstream without a Raft. Cell 121:815-816.

Lipardi C, Nitsch L, and Zurzolo C. 2000. Detergent-insoluble GPI-anchored proteins are apically sorted in fischer rat thyroid cells, but interference with cholesterol or sphingolipids differentially affects detergent insolubility and apical sorting. Mol Biol Cell 11:531-542.

Lisanti MP, Sargiacomo M, Graeve L, Saltiel AR, and Rodriguez-Boulan E. 1988. Polarized apical distribution of glycosyl-phosphatidylinositol-anchored proteins in a renal epithelial cell line. Proc Natl Acad Sci U S A 85:9557-9561.

Liscovitch M, Chalifa V, Pertile P, Chen CS, and Cantley LC. 1994. Novel function of phosphatidylinositol 4,5-bisphosphate as a cofactor for brain membrane phospholipase D. J Biol Chem 269:21403-21406.

Liscovitch M, Czarny M, Fiucci G, and Tang X. 2000. Phospholipase D: molecular and cell biology of a novel gene family. Biochem J 345 Pt 3:401-415.

Locke D, Liu J, and Harris AL. 2005. Lipid rafts prepared by different methods contain different connexin channels, but gap junctions are not lipid rafts. Biochemistry 44:13027-13042.

Lockwich TP, Liu X, Singh BB, Jadlowiec J, Weiland S, and Ambudkar IS. 2000. Assembly of Trp1 in a signaling complex associated with caveolin-scaffolding lipid raft domains. J Biol Chem 275:11934-11942.

Lommerse PH, Spaink HP, and Schmidt T. 2004. In vivo plasma membrane organization: results of biophysical approaches. Biochim Biophys Acta 1664:119-131.

London E and Brown DA. 2000. Insolubility of lipids in triton X-100: physical origin and relationship to sphingolipid/cholesterol membrane domains (rafts). Biochim Biophys Acta 1508:182-195.

Lou Z, Jevremovic D, Billadeau DD, and Leibson PJ. 2000. A balance between positive and negative signals in cytotoxic lymphocytes regulates the polarization of lipid rafts during the development of cell-mediated killing. J Exp Med 191:347-354.

Louvet-Vallee S. 2000. ERM proteins: from cellular architecture to cell signaling. Biol Cell 92:305-316.

Maestes DC, Potter RM, and Prossnitz ER. 1999. Differential phosphorylation paradigms dictate desensitization and internalization of the N-formyl peptide receptor. J Biol Chem 274:29791-29795.

Magee T, Pirinen N, Adler J, Pagakis SN, and Parmryd I. 2002. Lipid rafts: cell surface platforms for T cell signaling. Biol Res 35:127-131.

Mahmudi-Azer S and van Eeden SF. 2003. Neutrophil 'connectivity': key to neutrophil-mediated tissue injury? Crit Care 7:285-287.

Mahon MJ, Donowitz M, Yun CC, and Segre GV. 2002. Na(+)/H(+) exchanger regulatory factor 2 directs parathyroid hormone 1 receptor signalling. Nature 417:858-861.

Malech HL, Root RK, and Gallin JI. 1977. Structural analysis of human neutrophil migration. Centriole, microtubule, and microfilament orientation and function during chemotaxis. J Cell Biol 75:666-693.

Manes S, Mira E, Gomez-Mouton C, Lacalle RA, Keller P, Labrador JP, and Martinez A. 1999. Membrane raft microdomains mediate front-rear polarity in migrating cells. EMBO J 18:6211-6220.

Manev V, Maneva A, and Sirakov L. 1998. Effect of lactoferrin on the phagocytic activity of polymorphonuclear leucocytes isolated from blood of patients with autoimmune diseases and Staphylococcus aureus allergy. Adv Exp Med Biol 443:321-330.

Mansfield PJ, Carey SS, Hinkovska-Galcheva V, Shayman JA, and Boxer LA. 2004. Ceramide inhibition of phospholipase D and its relationship to RhoA and ARF1 translocation in GTP gamma S-stimulated polymorphonuclear leukocytes. Blood 103:2363-2368.

Mansfield PJ, Hinkovska-Galcheva V, Carey SS, Shayman JA, and Boxer LA. 2002. Regulation of polymorphonuclear leukocyte degranulation and oxidant production by ceramide through inhibition of phospholipase D. Blood 99:1434-1441.

Marcil J, Harbour D, Naccache PH, and Bourgoin S. 1997. Human phospholipase D1 can be tyrosine-phosphorylated in HL-60 granulocytes. J Biol Chem 272:20660-20664.

Marinetti GV and Love R. 1976. Differential reaction of cell membrane phospholipids and proteins with chemical probes. Chem Phys Lipids 16:239-254.

Marshall J, Krump E, Lindsay T, Downey G, Ford DA, Zhu P, Walker P, and Rubin B. 2000. Involvement of cytosolic phospholipase A2 and secretory phospholipase A2 in arachidonic acid release from human neutrophils. J Immunol 164:2084-2091.

Martin OC and Pagano RE. 1987. Transbilayer movement of fluorescent analogs of phosphatidylserine and phosphatidylethanolamine at the plasma membrane of cultured cells. Evidence for a protein-mediated and ATP-dependent process(es). J Biol Chem 262:5890-5898.

Martin TF. 2001. PI(4,5)P(2) regulation of surface membrane traffic. Curr Opin Cell Biol 13:493-499.

Matecki A and Pawelczyk T. 1997. Regulation of phospholipase C delta1 by sphingosine. Biochim Biophys Acta 1325:287-296.

Matter K and Mellman I. 1994. Mechanisms of cell polarity: sorting and transport in epithelial cells. Curr Opin Cell Biol 6:545-554.

McClenahan DJ, Evanson OA, and Weiss DJ. 2002. In vitro evaluation of the role of platelet-activating factor and interleukin-8 in Mannheimia haemolytica-induced bovine pulmonary endothelial cell injury. Am J Vet Res 63:394-401.

McColl SR, Beauseigle D, Gilbert C, and Naccache PH. 1990. Priming of the human neutrophil respiratory burst by granulocyte-macrophage colony-stimulating factor and tumor necrosis factor-alpha involves regulation at a post-cell surface receptor level. Enhancement of the effect of agents which directly activate G proteins. J Immunol 145:3047-3053.

McEvoy L, Williamson P, and Schlegel RA. 1986. Membrane phospholipid asymmetry as a determinant of erythrocyte recognition by macrophages. Proc Natl Acad Sci U S A 83:3311-3315.

McKay DA, Kusel JR, and Wilkinson PC. 1991. Studies of chemotactic factor-induced polarity in human neutrophils. Lipid mobility, receptor distribution and the time-sequence of polarization. J Cell Sci 100 (Pt 3):473-479.

McLeish KR, Gierschik P, Schepers T, Sidiropoulos D, and Jakobs KH. 1989. Evidence that activation of a common G-protein by receptors for leukotriene B4 and N-formylmethionyl-leucyl-phenylalanine in HL-60 cells occurs by different mechanisms. Biochem J 260:427-434.

McMurray RW and Hardy KJ. 2002. Cox-2 inhibitors: today and tomorrow. Am J Med Sci 323:181-189.

Megha and London E. 2004. Ceramide selectively displaces cholesterol from ordered lipid domains (rafts): implications for lipid raft structure and function. J Biol Chem 279:9997-10004.

Meiri KF. 2004. Membrane/cytoskeleton communication. Subcell Biochem 37:247-282.

Meiri KF. 2005. Lipid rafts and regulation of the cytoskeleton during T cell activation. Philos Trans R Soc Lond B Biol Sci 360:1663-1672.

Mejean C, Lebart MC, Roustan C, and Benyamin Y. 1995. Inhibition of actin-dystrophin interaction by inositide phosphate. Biochem Biophys Res Commun 210:152-158.

Melamed I, Downey GP, Aktories K, and Roifman CM. 1991. Microfilament assembly is required for antigen-receptor-mediated activation of human B lymphocytes. J Immunol 147:1139-1146.

Melamed I and Gelfand EW. 1999. Microfilament assembly is involved in B-cell apoptosis. Cell Immunol 194:136-142.

Mendez AJ, Lin G, Wade DP, Lawn RM, and Oram JF. 2000. Membrane lipid domains distinct from cholesterol/sphingomyelin-rich rafts are involved in the ABCA1-mediated lipid secretory pathway. J Biol Chem 101.

Menon AK, Holowka D, and Baird B. 1984. Small oligomers of immunoglobulin E (IgE) cause large-scale clustering of IgE receptors on the surface of rat basophilic leukemia cells. J Cell Biol 98:577-583.

Meyer zum Bueschenfelde CO, Unternaehrer J, Mellman I, and Bottomly K. 2004. Regulated recruitment of MHC class II and costimulatory molecules to lipid rafts in dendritic cells. J Immunol 173:6119-6124.

Meyer T, Wellner-Kienitz MC, Biewald A, Bender K, Eickel A, and Pott L. 2001. Depletion of phosphatidylinositol 4,5-bisphosphate by activation of phospholipase C-coupled receptors causes slow inhibition but not desensitization of G protein-gated inward rectifier K+ current in atrial myocytes. J Biol Chem 276:5650-5658.

Miettinen HM, Mills JS, Gripentrog JM, Dratz EA, Granger BL, and Jesaitis AJ. 1997. The ligand binding site of the formyl peptide receptor maps in the transmembrane region. J Immunol 159:4045-4054.

Miotti S, Bagnoli M, Tomassetti A, Colnaghi MI, and Canevari S. 2000. Interaction of folate receptor with signaling molecules lyn and G(alpha)(i-3) in detergent-resistant complexes from the ovary carcinoma cell line IGROV1. J Cell Sci 113 Pt 2:349-357.

Mocsai A, Ligeti E, Lowell CA, and Berton G. 1999. Adhesion-dependent degranulation of neutrophils requires the Src family kinases Fgr and Hck. J Immunol 162:1120-1126.

Moffett S, Brown DA, and Linder ME. 2000. Lipid-dependent targeting of G proteins into rafts. J Biol Chem 275:2191-2198.

Moniakis J, Funamoto S, Fukuzawa M, Meisenhelder J, Araki T, Abe T, Meili R, Hunter T, Williams J, and Firtel RA. 2001. An SH2-domain-containing kinase negatively regulates the phosphatidylinositol-3 kinase pathway. Genes Dev 15:687-698.

Moreau C, Sulpice JC, Devaux PF, and Zachowski A. 1997. Drug-induced transmembrane lipid scrambling in erythrocytes and in liposomes requires the presence of polyanionic phospholipids. Mol Membr Biol 14:5-12.

Mouritsen OG and Bloom M. 1993. Models of lipid-protein interactions in membranes. Annu Rev Biophys Biomol Struct 22:145-171.

Mukherjee G, Quinn MT, Linner JG, and Jesaitis AJ. 1994. Remodeling of the plasma membrane after stimulation of neutrophils with f-Met-Leu-Phe and dihydrocytochalasin B: identification of membrane subdomains containing NADPH oxidase activity. J Leukoc Biol 55:685-694.

Mullins RD. 2000. How WASP-family proteins and the Arp2/3 complex convert intracellular signals into cytoskeletal structures. Curr Opin Cell Biol 12:91-96.

Mullmann TJ, Cheewatrakoolpong B, Anthes JC, Siegel MI, Egan RW, and Billah MM. 1993. Phospholipase C and phospholipase D are activated independently of each other in chemotactic peptide-stimulated human neutrophils. J Leukoc Biol 53:630-635.

Murakami K, Okajima K, Harada N, Isobe H, and Okabe H. 1998. Rebamipide prevents indomethacin-induced gastric mucosal lesion formation by inhibiting activation of neutrophils in rats. Dig Dis Sci 43:139S-142S.

Musch A, Xu H, Shields D, and Rodriguez-Boulan E. 1996. Transport of vesicular stomatitis virus G protein to the cell surface is signal mediated in polarized and nonpolarized cells. J Cell Biol 133:543-558.

N'Diaye EN, Darzacq X, Astarie-Dequeker C, Daffe M, Calafat J, and Maridonneau-Parini I. 1998. Fusion of azurophil granules with phagosomes and activation of the tyrosine kinase Hck are specifically inhibited during phagocytosis of mycobacteria by human neutrophils. J Immunol 161:4983-4991.

Naga Prasad SV, Esposito G, Mao L, Koch WJ, and Rockman HA. 2000. Gbetagamma-dependent phosphoinositide 3-kinase activation in hearts with in vivo pressure overload hypertrophy. J Biol Chem 275:4693-4698.

Nagano C, Azuma A, Ishiyama H, Sekiguchi K, Imagawa K, and Kikuchi M. 2001. Rebamipide suppresses formyl-methionyl-leucyl-phenylalanine (fMLP)-induced superoxide production by inhibiting fMLP-receptor binding in human neutrophils. J Pharmacol Exp Ther 297:388-394.

Nagano S, Otsuka T, Niiro H, Yamaoka K, Arinobu Y, Ogami E, Akahoshi M, Inoue Y, Miyake K, Nakashima H, Niho Y, and Harada M. 2002. Molecular mechanisms of lipopolysaccharide-induced cyclooxygenase-2 expression in human neutrophils: involvement of the mitogen-activated protein kinase pathway and regulation by anti-inflammatory cytokines. Int Immunol 14:733-740.

Nagaoka R, Abe H, and Obinata T. 1996. Site-directed mutagenesis of the phosphorylation site of cofilin: its role in cofilin-actin interaction and cytoplasmic localization. Cell Motil Cytoskeleton 35:200-209.

Nakamoto H, Hashikita Y, Itabashi A, Kobayashi T, and Suzuki H. 2004. Changes in the organisms of resistant peritonitis in patients on continuous ambulatory peritoneal dialysis. Adv Perit Dial 20:52-57.

Nakamura T, Suchard SJ, Abe A, Shayman JA, and Boxer LA. 1994. Role of diradylglycerol formation in H2O2 and lactoferrin release in adherent human polymorphonuclear leukocytes. J Leukoc Biol 56:105-109.

Niedel J. 1981. Detergent solubilization of the formyl peptide chemotactic receptor. Strategy based on covalent affinity labeling. J Biol Chem 256:9295-9299.

Nielander HB, Onofri F, Schaeffer E, Menegon A, Fesce R, Valtorta F, Greengard P, and Benfenati F. 1997. Phosphorylation-dependent effects of synapsin IIa on actin polymerization and network formation. Eur J Neurosci 9:2712-2722.

Niggli V. 2003. Signaling to migration in neutrophils: importance of localized pathways. Int J Biochem Cell Biol 35:1619-1638.

Nika K, Charvet C, Williams S, Tautz L, Bruckner S, Rahmouni S, Bottini N, Schoenberger SP, Baier G, Altman A, and Mustelin T. 2006. Lipid raft targeting of hematopoietic protein tyrosine phosphatase by protein kinase C theta-mediated phosphorylation. Mol Cell Biol 26:1806-1816.

Nikolopoulos SN and Turner CE. 2000. Actopaxin, a New Focal Adhesion Protein That Binds Paxillin LD Motifs and Actin and Regulates Cell Adhesion. J Cell Biol 151:1435-1448.

Niv H, Gutman O, Kloog Y, and Henis YI. 2002. Activated K-Ras and H-Ras display different interactions with saturable nonraft sites at the surface of live cells. J Cell Biol 157:865-872.

Noegel AA and Schleicher M. 2000. The actin cytoskeleton of Dictyostelium: a story told by mutants. J Cell Sci 113:759-766.

Nomoto K, Tomita N, Miyake M, Xhu DB, LoGerfo PR, and Weinstein IB. 1998. Growth inhibition, enhancement of intercellular adhesion, and increased expression of carcinoembryonic antigen by overexpression of phosphoinositides-specific phospholipase C beta 1 in LS174T human colon adenocarcinoma cell line. Jpn J Cancer Res 89:1257-1266.

Nusrat A, Parkos CA, Verkade P, Foley CS, Liang TW, Innis-Whitehouse W, Eastburn KK, and Madara JL. 2000. Tight junctions are membrane microdomains. J Cell Sci 113:4-81.

Nusse O and Lindau M. 1988. The dynamics of exocytosis in human neutrophils. J Cell Biol 107:2117-2123.

Nutikka A and Lingwood C. 2004. Generation of receptor-active, globotriaosyl ceramide/cholesterol lipid 'rafts' in vitro : A new assay to define factors affecting glycosphingolipid receptor activity. Glycoconj J 20:33-38.

O'Flaherty JT, Redman JF, Jacobson DP, and Rossi AG. 1990. Stimulation and priming of protein kinase C translocation by a Ca2+ transient-independent mechanism. Studies in human neutrophils challenged with platelet-activating factor and other receptor agonists. J Biol Chem 265:21619-21623.

O'Neill JP and Hsie AW. 1978. Growth arrest of Chinese hamster ovary cells in serum-free medium: changes in cell morphology and the intracellular and prostaglandin-induced levels of cyclic AMP. J Cyclic Nucleotide Res 4:169-174.

Oliferenko S, Paiha K, Harder T, Gerke V, Schwarzler C, Schwarz H, Beug H, Gunthert U, and Huber LA. 1999. Analysis of CD44-containing lipid rafts: Recruitment of annexin II and stabilization by the actin cytoskeleton. J Cell Biol 146:843-854.

Oliver JM. 1978. Cell biology of leukocyte abnormalities--membrane and cytoskeletal function in normal and defective cells. A review. Am J Pathol 93:221-270.

Oliver JM and Berlin RD. 1982. Mechanisms that regulate the structural and functional architecture of cell surfaces. Int Rev Cytol 74:55-94.

Olorundare OE, Simmons SR, and Albrecht RM. 1993. Evidence for two mechanisms of ligand-receptor movement on surface-activated platelets. Eur J Cell Biol 60:131-145.

Olson SC and Lambeth JD. 1996. Biochemistry and cell biology of phospholipase D in human neutrophils. Chem Phys Lipids 80:3-19.

Omidvar N, Wang EC, Brennan P, Longhi MP, Smith RA, and Morgan BP. 2006. Expression of Glycosylphosphatidylinositol-Anchored CD59 on Target Cells Enhances Human NK Cell-Mediated Cytotoxicity. J Immunol 176:2915-2923.

Onaran HO, Costa T, and Rodbard D. 1993. Beta gamma subunits of guanine nucleotide-binding proteins and regulation of spontaneous receptor activity: thermodynamic model for the interaction between receptors and guanine nucleotide-binding protein subunits. Mol Pharmacol 43:245-256.

Ortmeyer J and Mohsenin V. 1993. Glucose suppresses superoxide generation in normal neutrophils: interference in phospholipase D activation. Am J Physiol 264:C402-C410.

Ostermeyer AG, Beckrich BT, Ivarson KA, Grove KE, and Brown DA. 1999. Glycosphingolipids are not essential for formation of detergent-resistant membrane rafts in melanoma cells. methyl-beta-cyclodextrin does not affect cell surface transport of a GPI-anchored protein. J Biol Chem 274:34459-34466.

Ostrom RS, Post SR, and Insel PA. 2000. Stoichiometry and compartmentation in G protein-coupled receptor signaling: implications for therapeutic interventions involving G(s). J Pharmacol Exp Ther 294:407-412.

Pankov R, Endo Y, Even-Ram S, Araki M, Clark K, Cukierman E, Matsumoto K, and Yamada KM. 2005. A Rac switch regulates random versus directionally persistent cell migration. J Cell Biol 170:793-802.

Parasassi T, Gratton E, Zajicek H, Levi M, and Yu W. 1999. Detecting membrane lipid microdomains by two-photon fluorescence microscopy. IEEE Eng Med Biol Mag 18:92-99.

Parent CA. 2004. Making all the right moves: chemotaxis in neutrophils and Dictyostelium. Curr Opin Cell Biol 16:4-13.

Parent CA, Blacklock BJ, Froehlich WM, Murphy DB, and Devreotes PN. 1998. G protein signaling events are activated at the leading edge of chemotactic cells. Cell 95:81-91.

Parent CA and Devreotes PN. 1999. A cell's sense of direction. Science 284:765-770.

Park KC, Rivero F, Meili R, Lee S, Apone F, and Firtel RA. 2004. Rac regulation of chemotaxis and morphogenesis in Dictyostelium. EMBO J 23:4177-4189.

Parkos CA, Cochrane CG, Schmitt M, and Jesaitis AJ. 1985. Regulation of the oxidative response of human granulocytes to chemoattractants. No evidence for stimulated traffic of redox enzymes between endo and plasma membranes. J Biol Chem 260:6541-6547.

Parmley RT, Rice WG, Kinkade JM, Jr., Gilbert C, and Barton JC. 1987. Peroxidase-containing microgranules in human neutrophils: physical, morphological, cytochemical, and secretory properties. Blood 70:1630-1638.

Parmryd I, Adler J, Patel R, and Magee AI. 2003. Imaging metabolism of phosphatidylinositol 4,5-bisphosphate in T-cell GM1-enriched domains containing Ras proteins. Exp Cell Res 285:27-38.

Parpal S, Gustavsson J, and Stralfors P. 1995. Isolation of phosphooligosaccharide/phosphoinositol glycan from caveolae and cytosol of insulin-stimulated cells. J Cell Biol 131:125-135.

Parton RG and Richards AA. 2003. Lipid rafts and caveolae as portals for endocytosis: new insights and common mechanisms. Traffic 4:724-738.

Patterson GM, Smith CD, Kimura LH, Britton BA, and Carmeli S. 1993. Action of tolytoxin on cell morphology, cytoskeletal organization, and actin polymerization. Cell Motil Cytoskeleton 24:39-48.

Payrastre B, Nievers M, Boonstra J, Breton M, Verkleij AJ, and Van Bergen en Henegouwen PM. 1992. A differential location of phosphoinositide kinases, diacylglycerol kinase, and phospholipase C in the nuclear matrix. J Biol Chem 267:5078-5084.

Pediconi MF, Gallegos CE, Los Santos EB, and Barrantes FJ. 2004. Metabolic cholesterol depletion hinders cell-surface trafficking of the nicotinic acetylcholine receptor. Neuroscience 128:239-249.

Pelletier R, Peter J, Antin C, Gonzalez C, Wood L, and Walsh TJ. 2000. Emergence of resistance of Candida albicans to clotrimazole in human immunodeficiency virus-infected children: in vitro and clinical correlations. J Clin Microbiol 38:1563-1568.

Pellme S, Morgelin M, Tapper H, Mellqvist UH, Dahlgren C, and Karlsson A. 2006. Localization of human neutrophil interleukin-8 (CXCL-8) to organelle(s) distinct from the classical granules and secretory vesicles. J Leukoc Biol 79:564-573.

Peranen J, Auvinen P, Virta H, Wepf R, and Simons K. 1996. Rab8 promotes polarized membrane transport through reorganization of actin and microtubules in fibroblasts. J Cell Biol 135:153-167.

Perez HD, Elfman F, Marder S, Lobo E, and Ives HE. 1989. Formyl peptide-induced chemotaxis of human polymorphonuclear leukocytes does not require either marked changes in cytosolic calcium or specific granule discharge. Role of formyl peptide receptor reexpression (or recycling). J Clin Invest 83:1963-1970.

Perez HD, Kelly E, Elfman F, Armitage G, and Winkler J. 1991. Defective polymorphonuclear leukocyte formyl peptide receptor(s) in juvenile periodontitis. J Clin Invest 87:971-976.

Perroy J, Gutierrez GJ, Coulon V, Bockaert J, Pin JP, and Fagni L. 2001. The C terminus of the metabotropic glutamate receptor subtypes 2 and 7 specifies the receptor signaling pathways. J Biol Chem 276:45800-45805.

Perskvist N, Roberg K, Kulyte A, and Stendahl O. 2002. Rab5a GTPase regulates fusion between pathogen-containing phagosomes and cytoplasmic organelles in human neutrophils. J Cell Sci 115:1321-1330.

Pertile P, Liscovitch M, Chalifa V, and Cantley LC. 1995. Phosphatidylinositol 4,5-bisphosphate synthesis is required for activation of phospholipase D in U937 cells. J Biol Chem 270:5130-5135.

Peters KR, Carley WW, and Palade GE. 1985. Endothelial plasmalemmal vesicles have a characteristic striped bipolar surface structure. J Cell Biol 101:2233-2238.

Petrequin PR, Todd RF, III, Smolen JE, and Boxer LA. 1986. Expression of specific granule markers on the cell surface of neutrophil cytoplasts. Blood 67:1119-1125.

Petrie RJ, Schnetkamp PP, Patel KD, Awasthi-Kalia M, and Deans JP. 2000. Transient translocation of the B cell receptor and Src homology 2 domain-containing inositol phosphatase to lipid rafts: evidence toward a role in calcium regulation. J Immunol 165:1220-1227.

Pierini LM, Eddy RJ, Fuortes M, Seveau S, Casulo C, and Maxfield FR. 2003. Membrane lipid organization is critical for human neutrophil polarization. J Biol Chem 278:10831-10841.

Pierini LM and Maxfield FR. 2001. Flotillas of lipid rafts fore and aft. Proc Natl Acad Sci U S A 98:9471-9473.

Pike LJ and Miller JM. 1998. Cholesterol depletion delocalizes phosphatidylinositol bisphosphate and inhibits hormone-stimulated phosphatidylinositol turnover. J Biol Chem 273:22298-22304.

Pike MC, Jakoi L, McPhail LC, and Snyderman R. 1986. Chemoattractant-mediated stimulation of the respiratory burst in human polymorphonuclear leukocytes may require appearance of protein kinase activity in the cells' particulate fraction. Blood 67:909-913.

Pitcher JA, Touhara K, Payne ES, and Lefkowitz RJ. 1995. Pleckstrin homology domain-mediated membrane association and activation of the beta-adrenergic receptor kinase requires coordinate interaction with G beta gamma subunits and lipid. J Biol Chem 270:11707-11710.

Pizzo P and Viola A. 2004. Lipid rafts in lymphocyte activation. Microbes Infect 6:686-692.

Polozova A and Litman BJ. 2000. Cholesterol dependent recruitment of di22:6-PC by a G protein-coupled receptor into lateral domains. Biophys J 79:2632-2643.

Pradhan D and Morrow J. 2002. The spectrin-ankyrin skeleton controls CD45 surface display and interleukin-2 production. Immunity 17:303-315.

Premont RT and Hall RA. 2002. Identification of novel G protein-coupled receptor-interacting proteins. Methods Enzymol 343:611-621.

Procko E and McColl SR. 2005. Leukocytes on the move with phosphoinositide 3-kinase and its downstream effectors. Bioessays 27:153-163.

Provost JJ, Fudge J, Israelit S, Siddiqi AR, and Exton JH. 1996. Tissue-specific distribution and subcellular distribution of phospholipase D in rat: evidence for distinct RhoA- and ADP-ribosylation factor (ARF)-regulated isoenzymes. Biochem J 319 (Pt 1):285-291.

Pryzwansky KB, Schliwa M, and Boxer LA. 1985. Microtubule organization of unstimulated and stimulated adherent human neutrophils in Chediak-Higashi syndrome. Blood 66:1398-1403.

Ptasznik A, Traynor-Kaplan A, and Bokoch GM. 1995. G protein-coupled chemoattractant receptors regulate Lyn tyrosine kinase.Shc adapter protein signaling complexes. J Biol Chem 270:19969-19973.

Pyenta PS, Holowka D, and Baird B. 2001. Cross-correlation analysis of inner-leaflet-anchored green fluorescent protein co-redistributed with IgE receptors and outer leaflet lipid raft components. Biophys J 80:2120-2132.

Pyne S and Pyne NJ. 2002. Sphingosine 1-phosphate signalling and termination at lipid phosphate receptors. Biochim Biophys Acta 1582:121-131.

Quehenberger O, Prossnitz ER, Cavanagh SL, Cochrane CG, and Ye RD. 1993. Multiple domains of the N-formyl peptide receptor are required for high-affinity ligand binding. Construction and analysis of chimeric N-formyl peptide receptors. J Biol Chem 268:18167-18175.

Quinn MT, Parkos CA, and Jesaitis AJ. 1989. The lateral organization of components of the membrane skeleton and superoxide generation in the plasma membrane of stimulated human neutrophils. Biochim Biophys Acta 987:83-94.

Quinn PJ. 2002. Plasma membrane phospholipid asymmetry. Subcell Biochem 36:39-60.

Rais S, Pedruzzi E, Dang MC, Giroud JP, Hakim J, and Perianin A. 1998. Priming of phosphatidic acid production by staurosporine in f-Met-Leu-Phe-stimulated human neutrophils--correlation with respiratory burst. Cell Signal 10:121-129.

Rane MJ, Carrithers SL, Arthur JM, Klein JB, and McLeish KR. 1997. Formyl peptide receptors are coupled to multiple mitogen-activated protein kinase cascades by distinct signal transduction pathways: role in activation of reduced nicotinamide adenine dinucleotide oxidase. J Immunol 159:5070-5078.

Reddy MK, Rabinowitz M, and Zak R. 1983. Stringent requirement for Ca2+ in the removal of Z-lines and alpha-actinin from isolated myofibrils by Ca2+-activated neutral proteinase. Biochem J 209:635-641.

Reumaux D, Kuijpers TW, Hordijk PL, Duthilleul P, and Roos D. 2003. Involvement of Fcgamma receptors and beta2 integrins in neutrophil activation by anti-proteinase-3 or anti-myeloperoxidase antibodies. Clin Exp Immunol 134:344-350.

Rey M, Vicente-Manzanares M, Viedma F, Yanez-Mo M, Urzainqui A, Barreiro O, Vazquez J, and Sanchez-Madrid F. 2002. Cutting edge: association of the motor protein nonmuscle myosin heavy chain-IIA with the C terminus of the chemokine receptor CXCR4 in T lymphocytes. J Immunol 169:5410-5414.

Richard JF, Petit L, Gibert M, Marvaud JC, Bouchaud C, and Popoff MR. 1999. Bacterial toxins modifying the actin cytoskeleton. Int Microbiol 2:185-194.

Richter J, Andersson T, and Olsson I. 1989. Effect of tumor necrosis factor and granulocyte/macrophage colony-stimulating factor on neutrophil degranulation. J Immunol 142:3199-3205.

Rickert P, Weiner OD, Wang F, Bourne HR, and Servant G. 2000. Leukocytes navigate by compass: roles of PI3Kgamma and its lipid products. Trends Cell Biol 10:466-473.

Ridyard MS and Robbins SM. 2003. Fibroblast growth factor-2-induced signaling through lipid raft-associated fibroblast growth factor receptor substrate 2 (FRS2). J Biol Chem 278:13803-13809.

Ritchie K, Iino R, Fujiwara T, Murase K, and Kusumi A. 2003. The fence and picket structure of the plasma membrane of live cells as revealed by single molecule techniques (Review). Mol Membr Biol 20:13-18.

Ritchie K and Kusumi A. 2004. Role of the membrane skeleton in creation of microdomains. Subcell Biochem 37:233-245.

Robertson SE, Young JD, Kitson S, Pitt A, Evans J, Roes J, Karaoglu D, Santora L, Ghayur T, Liew FY, Gracie JA, and McInnes IB. 2006. Expression and alternative processing of IL-18 inhuman neutrophils. Eur J Immunol 36:722-731.

Robinson JM, Kobayashi T, Seguchi H, and Takizawa T. 1999. Evaluation of neutrophil structure and function by electron microscopy: cytochemical studies. J Immunol Methods 232:169-178.

Rodionov VI, Gyoeva FK, Tanaka E, Bershadsky AD, Vasiliev JM, and Gelfand VI. 1993. Microtubule-dependent control of cell shape and pseudopodial activity is inhibited by the antibody to kinesin motor domain. J Cell Biol 123:1811-1820.

Rodriguez-Boulan E and Nelson WJ. 1989. Morphogenesis of the polarized epithelial cell phenotype. Science 245:718-725.

Rohatgi R, Ho HY, and Kirschner MW. 2000. Mechanism of N-WASP activation by CDC42 and phosphatidylinositol 4, 5-bisphosphate. J Cell Biol 150:1299-1310.

Rohatgi R, Ma L, Miki H, Lopez M, Kirchhausen T, Takenawa T, and Kirschner MW. 1999. The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly. Cell 97:221-231.

Round JL, Tomassian T, Zhang M, Patel V, Schoenberger SP, and Miceli MC. 2005. Dlgh1 coordinates actin polymerization, synaptic T cell receptor and lipid raft aggregation, and effector function in T cells. J Exp Med 201:419-430.

Rozelle AL, Machesky LM, Yamamoto M, Driessens MH, Insall RH, Roth MG, Luby-Phelps K, Marriott G, Hall A, and Yin HL. 2000. Phosphatidylinositol 4,5-bisphosphate induces actin-based movement of raft-enriched vesicles through WASP-Arp2/3. Curr Biol 10:311-320.

Rybin VO, Xu X, Lisanti MP, and Steinberg SF. 2000. Differential targeting of beta -adrenergic receptor subtypes and adenylyl cyclase to cardiomyocyte caveolae. A mechanism to functionally regulate the cAMP signaling pathway. J Biol Chem 275:41447-41457.

Sako Y and Kusumi A. 1994. Compartmentalized structure of the plasma membrane for receptor movements as revealed by a nanometer-level motion analysis. J Cell Biol 125:1251-1264.

Salzer W, Gerard C, and McCall C. 1987. Effect of an inhibitor of protein kinase C on human polymorphonuclear leukocyte degranulation. Biochem Biophys Res Commun 148:747-754.

Sargiacomo M, Sudol M, Tang Z, and Lisanti MP. 1993. Signal transducing molecules and glycosyl-phosphatidylinositol-linked proteins form a caveolin-rich insoluble complex in MDCK cells. J Cell Biol 122:789-807.

Saxton MJ. 1987. Lateral diffusion in an archipelago. The effect of mobile obstacles. Biophys J 52:989-997.

Saxton MJ. 1990c. Lateral diffusion in a mixture of mobile and immobile particles. A Monte Carlo study. Biophys J 58:1303-1306.

Saxton MJ. 1990a. The membrane skeleton of erythrocytes. A percolation model. Biophys J 57:1167-1177.

Saxton MJ. 1990b. The membrane skeleton of erythrocytes: models of its effect on lateral diffusion. Int J Biochem 22:801-809.

Schade AE and Levine AD. 2002. Lipid raft heterogeneity in human peripheral blood T lymphoblasts: a mechanism for regulating the initiation of TCR signal transduction. J Immunol 168:2233-2239.

Scheiffele P, Peranen J, and Simons K. 1995. N-glycans as apical sorting signals in epithelial cells. Nature 378:96-98.

Schlegel A, Volonte D, Engelman JA, Galbiati F, Mehta P, Zhang XL, Scherer PE, and Lisanti MP. 1998. Crowded little caves: structure and function of caveolae. Cell Signal 10:457-463.

Schmidt A and Hall MN. 1998. Signaling to the actin cytoskeleton. Annu Rev Cell Dev Biol 14:305-338.

Schmitz ML, Bacher S, and Dienz O. 2003. NF-kappaB activation pathways induced by T cell costimulation. FASEB J 17:2187-2193.

Schneider IC and Haugh JM. 2005. Quantitative elucidation of a distinct spatial gradient-sensing mechanism in fibroblasts. J Cell Biol 171:883-892.

Schnitzer JE, McIntosh DP, Dvorak AM, Liu J, and Oh P. 1995. Separation of caveolae from associated microdomains of GPI-anchored proteins. Science 269:1435-1439.

Schreiber A, Luft FC, and Kettritz R. 2004. Membrane proteinase 3 expression and ANCA-induced neutrophil activation. Kidney Int 65:2172-2183.

Schreiber S, Fleischer J, Breer H, and Boekhoff I. 2000. A possible role for caveolin as a signaling organizer in olfactory sensory membranes. J Biol Chem 275:24115-24123.

Schroder AK, von der OM, Fleischer D, Rink L, and Uciechowski P. 2005. Differential synthesis of two interleukin-1 receptor antagonist variants and interleukin-8 by peripheral blood neutrophils. Cytokine 32:246-253.

Schroeder RJ, Ahmed SN, Zhu Y, London E, and Brown DA. 1998. Cholesterol and sphingolipid enhance the Triton X-100 insolubility of glycosylphosphatidylinositol-anchored proteins by promoting the formation of detergent-insoluble ordered membrane domains. J Biol Chem 273:1150-1157.

Schroit AJ, Madsen JW, and Tanaka Y. 1985. In vivo recognition and clearance of red blood cells containing phosphatidylserine in their plasma membranes. J Biol Chem 260:5131-5138.

Schultz AM, Tsai SC, Kung HF, Oroszlan S, Moss J, and Vaughan M. 1987. Hydroxylamine-stable covalent linkage of myristic acid in G0 alpha, a guanine nucleotide-binding protein of bovine brain. Biochem Biophys Res Commun 146:1234-1239.

Schwartz RS, Chiu DT, and Lubin B. 1985. Plasma membrane phospholipid organization in human erythrocytes. Curr Top Hematol 5:63-112.

Schwencke C, Okumura S, Yamamoto M, Geng YJ, and Ishikawa Y. 1999. Colocalization of beta-adrenergic receptors and caveolin within the plasma membrane. J Cell Biochem 75:64-72.

Sciorra VA, Rudge SA, Wang J, McLaughlin S, Engebrecht J, and Morris AJ. 2002. Dual role for phosphoinositides in regulation of yeast and mammalian phospholipase D enzymes. J Cell Biol 159:1039-1049.

Scott RS, Sutton DA, and Jagirdar J. 2005. Lung infection due to opportunistic fungus, Phialemonium obovatum, in a bone marrow transplant recipient: an emerging infection with fungemia and Crohn disease-like involvement of the gastrointestinal tract. Ann Diagn Pathol 9:227-230.

Sechi AS and Wehland J. 2000. The actin cytoskeleton and plasma membrane connection: PtdIns(4,5)P(2) influences cytoskeletal protein activity at the plasma membrane. J Cell Sci 113 Pt 21:3685-3695.

Seely AJ, Pascual JL, and Christou NV. 2003. Science review: Cell membrane expression (connectivity) regulates neutrophil delivery, function and clearance. Crit Care 7:291-307.

Seguchi H and Kobayashi T. 2002. Study of NADPH oxidase-activated sites in human neutrophils. J Electron Microsc (Tokyo) 51:87-91.

Seifert R and Wenzel-Seifert K. 2001. Unmasking different constitutive activity of four chemoattractant receptors using Na+ as universal stabilizer of the inactive (R) state. Receptors Channels 7:357-369.

Seigneuret M and Devaux PF. 1984. ATP-dependent asymmetric distribution of spin-labeled phospholipids in the erythrocyte membrane: relation to shape changes. Proc Natl Acad Sci U S A 81:3751-3755.

Selvatici R, Falzarano S, Mollica A, and Spisani S. 2006. Signal transduction pathways triggered by selective formylpeptide analogues in human neutrophils. Eur J Pharmacol 534:1-11.

Sengelov H, Boulay F, Kjeldsen L, and Borregaard N. 1994a. Subcellular localization and translocation of the receptor for N-formylmethionyl-leucyl-phenylalanine in human neutrophils. Biochem J 299 (Pt 2):473-479.

Sengelov H, Kjeldsen L, and Borregaard N. 1993b. Control of exocytosis in early neutrophil activation. J Immunol 150:1535-1543.

Sengelov H, Kjeldsen L, Diamond MS, Springer TA, and Borregaard N. 1993a. Subcellular localization and dynamics of Mac-1 (alpha m beta 2) in human neutrophils. J Clin Invest 92:1467-1476.

Sengelov H, Kjeldsen L, Kroeze W, Berger M, and Borregaard N. 1994b. Secretory vesicles are the intracellular reservoir of complement receptor 1 in human neutrophils. J Immunol 153:804-810.

Sengelov H, Nielsen MH, and Borregaard N. 1992. Separation of human neutrophil plasma membrane from intracellular vesicles containing alkaline phosphatase and NADPH oxidase activity by free flow electrophoresis. J Biol Chem 267:14912-14917.

Serrador JM, Nieto M, Alonso-Lebrero JL, del Pozo MA, Calvo J, Furthmayr H, Schwartz-Albiez R, Lozano F, Gonzalez-Amaro R, Sanchez-Mateos P, and Sanchez-Madrid F. 1998. CD43 interacts with moesin and ezrin and regulates its redistribution to the uropods of T lymphocytes at the cell-cell contacts. Blood 91:4632-4644.

Servant G, Weiner OD, Herzmark P, Balla T, Sedat JW, and Bourne HR. 2000. Polarization of chemoattractant receptor signaling during neutrophil chemotaxis. Science 287:1037-1040.

Servant G, Weiner OD, Neptune ER, Sedat JW, and Bourne HR. 1999. Dynamics of a chemoattractant receptor in living neutrophils during chemotaxis. Mol Biol Cell 10:1163-1178.

Seveau S, Eddy RJ, Maxfield FR, and Pierini LM. 2001a. Cytoskeleton-dependent membrane domain segregation during neutrophil polarization. Mol Biol Cell 12:3550-3562.

Seveau S, Eddy RJ, Maxfield FR, and Pierini LM. 2001b. Cytoskeleton-dependent membrane domain segregation during neutrophil polarization. Mol Biol Cell 12:3550-3562.

Sheets ED, Holowka D, and Baird B. 1999b. Critical role for cholesterol in Lyn-mediated tyrosine phosphorylation of FcepsilonRI and their association with detergent-resistant membranes. J Cell Biol 145:877-887.

Sheets ED, Holowka D, and Baird B. 1999a. Membrane organization in immunoglobulin E receptor signaling. Curr Opin Chem Biol 3:95-99.

Sheets ED, Holowka D, and Baird B. 1999c. Membrane organization in immunoglobulin E receptor signaling. Curr Opin Chem Biol 3:95-99.

Sheetz MP. 1993. Glycoprotein motility and dynamic domains in fluid plasma membranes. Annu Rev Biophys Biomol Struct 22:417-431.

Shen L and Turner JR. 2005. Actin depolymerization disrupts tight junctions via caveolae-mediated endocytosis. Mol Biol Cell 16:3919-3936.

Sheng M and Kim E. 2000. The Shank family of scaffold proteins. J Cell Sci 113 (Pt 11):1851-1856.

Sheng M and Kim MJ. 2002. Postsynaptic signaling and plasticity mechanisms. Science 298:776-780.

Sheng M and Pak DT. 2000. Ligand-gated ion channel interactions with cytoskeletal and signaling proteins. Annu Rev Physiol 62:755-778.

Sheng M and Sala C. 2001. PDZ domains and the organization of supramolecular complexes. Annu Rev Neurosci 24:1-29.

Shimada Y, Inomata M, Suzuki H, Hayashi M, Abdul WA, and Ohno-Iwashita Y. 2005. Separation of a cholesterol-enriched microdomain involved in T-cell signal transduction. FEBS J 272:5454-5463.

Shinohara M, Terada Y, Iwamatsu A, Shinohara A, Mochizuki N, Higuchi M, Gotoh Y, Ihara S, Nagata S, Itoh H, Fukui Y, and Jessberger R. 2002. SWAP-70 is a guanine-nucleotide-exchange factor that mediates signalling of membrane ruffling. Nature 416:759-763.

Shogomori H and Brown DA. 2003. Use of detergents to study membrane rafts: the good, the bad, and the ugly. Biol Chem 384:1259-1263.

Siddiqui RA and English D. 2000. Phosphatidylinositol 3'-kinase-mediated calcium mobilization regulates chemotaxis in phosphatidic acid-stimulated human neutrophils. Biochim Biophys Acta 1483:161-173.

Sieper J and Braun J. 2001. New treatment options in ankylosing spondylitis: a role for anti-TNFalpha therapy. Ann Rheum Dis 60 Suppl 3:iii58-iii61.

Silvius JR. 2005. Partitioning of membrane molecules between raft and non-raft domains: insights from model-membrane studies. Biochim Biophys Acta 1746:193-202.

Silvius JR, del Giudice D, and Lafleur M. 1996. Cholesterol at different bilayer concentrations can promote or antagonize lateral segregation of phospholipids of differing acyl chain length. Biochemistry 35:15198-15208.

Simionescu N, Lupu F, and Simionescu M. 1983. Rings of membrane sterols surround the openings of vesicles and fenestrae, in capillary endothelium. J Cell Biol 97:1592-1600.

Simons K and Ikonen E. 1997. Functional rafts in cell membranes. Nature 387:569-572.

Simons K and van Meer G. 1988. Lipid sorting in epithelial cells. Biochemistry 27:6197-6202.

Simons M, Friedrichson T, Schulz JB, Pitto M, Masserini M, and Kurzchalia TV. 1999. Exogenous administration of gangliosides displaces GPI-anchored proteins from lipid microdomains in living cells. Mol Biol Cell 10:3187-3196.

Simson R, Yang B, Moore SE, Doherty P, Walsh FS, and Jacobson KA. 1998. Structural mosaicism on the submicron scale in the plasma membrane. Biophys J 74:297-308.

Singer SJ and Nicolson GL. 1972. The fluid mosaic model of the structure of cell membranes. Science 175:720-731.

Singh IN, Stromberg LM, Bourgoin SG, Sciorra VA, Morris AJ, and Brindley DN. 2001. Ceramide inhibition of mammalian phospholipase D1 and D2 activities is antagonized by phosphatidylinositol 4,5-bisphosphate. Biochemistry 40:11227-11233.

Skibbens JE, Roth MG, and Matlin KS. 1989. Differential extractability of influenza virus hemagglutinin during intracellular transport in polarized epithelial cells and nonpolar fibroblasts. J Cell Biol 108:821-832.

Smaby JM, Kulkarni VS, Momsen M, and Brown RE. 1996. The interfacial elastic packing interactions of galactosylceramides, sphingomyelins, and phosphatidylcholines. Biophys J 70:868-877.

Smart EJ, Ying YS, Mineo C, and Anderson RG. 1995. A detergent-free method for purifying caveolae membrane from tissue culture cells. Proc Natl Acad Sci U S A 92:10104-10108.

Smith RJ, Justen JM, and Sam LM. 1988a. Effects of a protein kinase C inhibitor, K-252a, on human polymorphonuclear neutrophil responsiveness. Biochem Biophys Res Commun 152:1497-1503.

Smith RJ, Sam LM, and Justen JM. 1988b. Diacylglycerols modulate human polymorphonuclear neutrophil responsiveness: effects on intracellular calcium mobilization, granule exocytosis, and superoxide anion production. J Leukoc Biol 43:411-419.

Smith RJ, Speziale SC, and Bowman BJ. 1985. Properties of interleukin-1 as a complete secretagogue for human neutrophils. Biochem Biophys Res Commun 130:1233-1240.

Sobue K, Morimoto K, Kanda K, Maruyama K, and Kakiuchi S. 1982. Reconstitution of Ca2+-sensitive gelation of actin filaments with filamin, caldesmon and calmodulin. FEBS Lett 138:289-292.

Somogyi G, Posta J, Buris L, and Varga M. 2006. Cyclodextrin (CD) complexes of cholesterol--their potential use in reducing dietary cholesterol intake. Pharmazie 61:154-156.

Song KS, Li S, Okamoto T, Quilliam LA, Sargiacomo M, and Lisanti MP. 1996. Co-purification and direct interaction of Ras with caveolin, an integral membrane protein of caveolae microdomains. Detergent-free purification of caveolae microdomains. J Biol Chem 271:9690-9697.

Spector AA and Yorek MA. 1985. Membrane lipid composition and cellular function. J Lipid Res 26:1015-1035.

Sproul TW, Malapati S, Kim J, and Pierce SK. 2000. Cutting edge: B cell antigen receptor signaling occurs outside lipid rafts in immature B cells. J Immunol 165:6020-6023.

Srinivasan S, Wang F, Glavas S, Ott A, Hofmann F, Aktories K, Kalman D, and Bourne HR. 2003. Rac and Cdc42 play distinct roles in regulating PI(3,4,5)P3 and polarity during neutrophil chemotaxis. J Cell Biol 160:375-385.

Stadel JM, DeLean A, and Lefkowitz RJ. 1980. A high affinity agonist . beta-adrenergic receptor complex is an intermediate for catecholamine stimulation of adenylate cyclase in turkey and frog erythrocyte membranes. J Biol Chem 255:1436-1441.

Stahelin RV, Ananthanarayanan B, Blatner NR, Singh S, Bruzik KS, Murray D, and Cho W. 2004. Mechanism of membrane binding of the phospholipase D1 PX domain. J Biol Chem 279:54918-54926.

Stan RV. 2005. Structure of caveolae. Biochim Biophys Acta 1746:334-348.

Sternfeld L, Th inverted question mF, and Schulz I. 2000. fMLP-induced arachidonic acid release in db-cAMP-differentiated HL-60 cells is independent of phosphatidylinositol-4, 5-bisphosphate-specific phospholipase C activation and cytosolic phospholipase A(2) activation. Arch Biochem Biophys 378:246-258.

Stossel TP, Hartwig JH, Janmey PA, and Kwiatkowski DJ. 1999. Cell crawling two decades after Abercrombie. Biochem Soc Symp 65:267-280.

Subczynski WK and Kusumi A. 2003. Dynamics of raft molecules in the cell and artificial membranes: approaches by pulse EPR spin labeling and single molecule optical microscopy. Biochim Biophys Acta 1610:231-243.

Suchard SJ, Hinkovska-Galcheva V, Mansfield PJ, Boxer LA, and Shayman JA. 1997. Ceramide inhibits IgG-dependent phagocytosis in human polymorphonuclear leukocytes. Blood 89:2139-2147.

Suchard SJ, Nakamura T, Abe A, Shayman JA, and Boxer LA. 1994. Phospholipase D-mediated diradylglycerol formation coincides with H2O2 and lactoferrin release in adherent human neutrophils. J Biol Chem 269:8063-8068.

Suchy SF and Nussbaum RL. 2002. The deficiency of PIP2 5-phosphatase in Lowe syndrome affects actin polymerization. Am J Hum Genet 71:1420-1427.

Sullivan SJ, Daukas G, and Zigmond SH. 1984. Asymmetric distribution of the chemotactic peptide receptor on polymorphonuclear leukocytes. J Cell Biol 99:1461-1467.

Sundaram M, Cook HW, and Byers DM. 2004. The MARCKS family of phospholipid binding proteins: regulation of phospholipase D and other cellular components. Biochem Cell Biol 82:191-200.

Sung TC, Altshuller YM, Morris AJ, and Frohman MA. 1999a. Molecular analysis of mammalian phospholipase D2. J Biol Chem 274:494-502.

Sung TC, Zhang Y, Morris AJ, and Frohman MA. 1999b. Structural analysis of human phospholipase D1. J Biol Chem 274:3659-3666.

Syrbu SI, Waterman WH, Molski TF, Nagarkatti D, Hajjar JJ, and Sha'afi RI. 1999. Phosphorylation of cytosolic phospholipase A2 and the release of arachidonic acid in human neutrophils. J Immunol 162:2334-2340.

Taborsky I and Dolnik V. 1977. Ability of human polymorhonuclear blood cells to produce interferon after induction with phage double-stranded RNA. Acta Virol 21:499-502.

Tadano-Aritomi K, Hikita T, Kubota M, Kasama T, Toma K, Hakomori SI, and Ishizuka I. 2003. Internal residue loss produced by rearrangement of a novel cationic glycosphingolipid, glyceroplasmalopsychosine, in collision-induced dissociation. J Mass Spectrom 38:715-722.

Takakuwa R, Kokai Y, Kojima T, Akatsuka T, Tobioka H, Sawada N, and Mori M. 2000. Uncoupling of gate and fence functions of MDCK cells by the actin-depolymerizing reagent mycalolide B. Exp Cell Res 257:238-244.

Tanaka S, Edberg JC, Chatham W, Fassina G, and Kimberly RP. 2003. Fc gamma RIIIb allele-sensitive release of alpha-defensins: anti-neutrophil cytoplasmic antibody-induced release of chemotaxins. J Immunol 171:6090-6096.

Tanimura M, Kobuchi H, Utsumi T, Yoshioka T, Kataoka S, Fujita Y, and Utsumi K. 1992. Neutrophil priming by granulocyte colony stimulating factor and its modulation by protein kinase inhibitors. Biochem Pharmacol 44:1045-1052.

Tanimura N, Saitoh SI, Kawano S, Kosugi A, and Miyake K. 2006. Palmitoylation of LAT contributes to its subcellular localization and stability. Biochem Biophys Res Commun 341:1177-1183.

Tansey MG, Baloh RH, Milbrandt J, and Johnson EM. 2000. GFRalpha-mediated localization of RET to lipid rafts is required for effective downstream signaling, differentiation, and neuronal survival. Neuron 25:611-623.

Taunton J. Actin filament nucleation by endosomes, lysosomes and secretory vesicles.

Terui T, Kahn RA, and Randazzo PA. 1994. Effects of acid phospholipids on nucleotide exchange properties of ADP-ribosylation factor 1. Evidence for specific interaction with phosphatidylinositol 4,5-bisphosphate. J Biol Chem 269:28130-28135.

Thiele B and Steinbach F. 1994. Dextran sulphate induces a PKC and actin independent internalisation of CD4. Immunol Lett 42:105-110.

Thivierge M, Parent JL, Stankova J, and Rola-Pleszczynski M. 1999. Modulation of formyl peptide receptor expression by IL-10 in human monocytes and neutrophils. J Immunol 162:3590-3595.

Tilley L, Cribier S, Roelofsen B, Op den Kamp JA, and van Deenen LL. 1986. ATP-dependent translocation of amino phospholipids across the human erythrocyte membrane. FEBS Lett 194:21-27.

Toker A. 2002. Phosphoinositides and signal transduction. Cell Mol Life Sci 59:761-779.

Tolias K and Carpenter CL. 2000. In vitro interaction of phosphoinositide-4-phosphate 5-kinases with Rac. Methods Enzymol 325:190-200.

Tolias KF, Cantley LC, and Carpenter CL. 1995. Rho family GTPases bind to phosphoinositide kinases. J Biol Chem 270:17656-17659.

Tolias KF, Couvillon AD, Cantley LC, and Carpenter CL. 1998. Characterization of a Rac1- and RhoGDI-associated lipid kinase signaling complex. Mol Cell Biol 18:762-770.

Tolias KF, Hartwig JH, Ishihara H, Shibasaki Y, Cantley LC, and Carpenter CL. 2000. Type Ialpha phosphatidylinositol-4-phosphate 5-kinase mediates Rac-dependent actin assembly. Curr Biol 10:153-156.

Tooze SA, Martens GJ, and Huttner WB. 2001. Secretory granule biogenesis: rafting to the SNARE. Trends Cell Biol 11:116-122.

Torres M and Ye RD. 1996. Activation of the mitogen-activated protein kinase pathway by fMet-leu-Phe in the absence of Lyn and tyrosine phosphorylation of SHC in transfected cells. J Biol Chem 271:13244-13249.

Truett AP, III, Verghese MW, Dillon SB, and Snyderman R. 1988. Calcium influx stimulates a second pathway for sustained diacylglycerol production in leukocytes activated by chemoattractants. Proc Natl Acad Sci U S A 85:1549-1553.

Truong HT, Ferrell JE, and Huestis WH. 1986. Sulfhydryl reducing agents and shape regulation in human erythrocytes. Blood 67:214-221.

Tsakiridis T, Tong P, Matthews B, Tsiani E, Bilan PJ, Klip A, and Downey GP. 1999. Role of the actin cytoskeleton in insulin action. Microsc Res Tech 47:79-92.

Tschesche H, Kopp C, Horl WH, and Hempelmann U. 1994. Inhibition of degranulation of polymorphonuclear leukocytes by angiogenin and its tryptic fragment. J Biol Chem 269:30274-30280.

Tse WY, Abadeh S, Jefferis R, Savage CO, and Adu D. 2000. Neutrophil FcgammaRIIIb allelic polymorphism in anti-neutrophil cytoplasmic antibody (ANCA)-positive systemic vasculitis. Clin Exp Immunol 119:574-577.

Tse WY, Abadeh S, McTiernan A, Jefferis R, Savage CO, and Adu D. 1999. No association between neutrophil FcgammaRIIa allelic polymorphism and anti-neutrophil cytoplasmic antibody (ANCA)-positive systemic vasculitis. Clin Exp Immunol 117:198-205.

Tsuji A and Ohnishi S. 1986. Restriction of the lateral motion of band 3 in the erythrocyte membrane by the cytoskeletal network: dependence on spectrin association state. Biochemistry 25:6133-6139.

Tsuji T and Miyama A. 1992. Chinese hamster ovary cells produce an enzyme that nicks heat-labile enterotoxin from enterotoxigenic Escherichia coli. Eur J Epidemiol 8:74-80.

Tumbarello M, Tacconelli E, Caponera S, Cauda R, and Ortona L. 1995. The impact of bacteraemia on HIV infection. Nine years experience in a large Italian university hospital. J Infect 31:123-131.

Turner NC, Wood LJ, Foster M, and Gueremy T. 1994. Effects of PAF, FMLP and opsonized zymosan on the release of ECP, elastase and superoxide from human granulocytes. Eur Respir J 7:934-940.

Twiss JL and van Minnen J. 2006. New insights into neuronal regeneration: the role of axonal protein synthesis in pathfinding and axonal extension. J Neurotrauma 23:295-308.

Twomey B, Muid RE, and Dale MM. 1990. The effect of putative protein kinase C inhibitors, K252a and staurosporine, on the human neutrophil respiratory burst activated by both receptor stimulation and post-receptor mechanisms. Br J Pharmacol 100:819-825.

Twomey BM, Clay K, and Dale MM. 1991. The protein kinase C inhibitor, K252a, inhibits superoxide production in human neutrophils activated by both PIP2-dependent and -independent mechanisms. Biochem Pharmacol 41:1449-1454.

Tzeng DY, Deuel TF, Huang JS, Senior RM, Boxer LA, and Baehner RL. 1984. Platelet-derived growth factor promotes polymorphonuclear leukocyte activation. Blood 64:1123-1128.

Tzima E, Trotter PJ, Orchard MA, and Walker JH. 2000. Annexin V relocates to the platelet cytoskeleton upon activation and binds to a specific isoform of actin. Eur J Biochem 267:4720-4730.

Ueno H, Sasaki K, Honda H, Nakamoto T, Yamagata T, Miyagawa K, Mitani K, Yazaki Y, and Hirai H. 1998. c-Cbl is tyrosine-phosphorylated by interleukin-4 and enhances mitogenic and survival signals of interleukin-4 receptor by linking with the phosphatidylinositol 3'-kinase pathway. Blood 91:46-53.

Uhing RJ, Prpic V, Hollenbach PW, and Adams DO. 1989. Involvement of protein kinase C in platelet-activating factor-stimulated diacylglycerol accumulation in murine peritoneal macrophages. J Biol Chem 264:9224-9230.

Ulloa-Aguirre A, Stanislaus D, Janovick JA, and Conn PM. 1999. Structure-activity relationships of G protein-coupled receptors. Arch Med Res 30:420-435.

van Haastert PJ and Kuwayama H. 1997. cGMP as second messenger during Dictyostelium chemotaxis. FEBS Lett 410:25-28.

van Meer G. 1989. Lipid traffic in animal cells. Annu Rev Cell Biol 5:247-275.

van Rheenen J, Achame EM, Janssen H, Calafat J, and Jalink K. 2005. PIP2 signaling in lipid domains: a critical re-evaluation. EMBO J 24:1664-1673.

Varma R and Mayor S. 1998. GPI-anchored proteins are organized in submicron domains at the cell surface. Nature 394:798-801.

Verghese MW, Charles L, Jakoi L, Dillon SB, and Snyderman R. 1987. Role of a guanine nucleotide regulatory protein in the activation of phospholipase C by different chemoattractants. J Immunol 138:4374-4380.

Verghese MW, Smith CD, and Snyderman R. 1986. Role of guanine nucleotide regulatory protein in polyphosphoinositide degradation and activation of phagocytic leukocytes by chemoattractants. J Cell Biochem 32:59-69.

Verkade P and Simons K. 1997. Robert Feulgen Lecture 1997. Lipid microdomains and membrane trafficking in mammalian cells. Histochem Cell Biol 108:211-220.

Vickers JD, Kinlough-Rathbone RL, and Mustard JF. 1987. Phosphatidylinositol 4,5-bisphosphate is selectively retained by platelet-fibrin clots formed by thrombin. Biochem J 245:649-653.

Vieira OV, Bucci C, Harrison RE, Trimble WS, Lanzetti L, Gruenberg J, Schreiber AD, Stahl PD, and Grinstein S. 2003. Modulation of Rab5 and Rab7 recruitment to phagosomes by phosphatidylinositol 3-kinase. Mol Cell Biol 23:2501-2514.

Villalba M, Bi K, Hu J, Altman Y, Bushway P, Reits E, Neefjes J, Baier G, Abraham RT, and Altman A. 2002. Translocation of PKC[theta] in T cells is mediated by a nonconventional, PI3-K- and Vav-dependent pathway, but does not absolutely require phospholipase C. J Cell Biol 157:253-263.

Villar AV, Alonso A, Paneda C, Varela-Nieto I, Brodbeck U, and Goni FM. 1999. Towards the in vitro reconstitution of caveolae. Asymmetric incorporation of glycosylphosphatidylinositol (GPI) and gangliosides into liposomal membranes. FEBS Lett 457:71-74.

Vines CM, Revankar CM, Maestas DC, LaRusch LL, Cimino DF, Kohout TA, Lefkowitz RJ, and Prossnitz ER. 2003. N-formyl peptide receptors internalize but do not recycle in the absence of arrestins. J Biol Chem 278:41581-41584.

Vinggaard AM, Provost JJ, Exton JH, and Hansen HS. 1997. Arf and RhoA regulate both the cytosolic and the membrane-bound phospholipase D from human placenta. Cell Signal 9:189-196.

Vissenberg K, Quelo AH, Van Gestel K, Olyslaegers G, and Verbelen JP. 2000. From hormone signal, via the cytoskeleton, to cell growth in single cells of tobacco. Cell Biol Int 24:343-349.

Vissers MC, Day WA, and Winterbourn CC. 1985. Neutrophils adherent to a nonphagocytosable surface (glomerular basement membrane) produce oxidants only at the site of attachment. Blood 66:161-166.

Walter RJ and Marasco WA. 1984. Localization of chemotactic peptide receptors on rabbit neutrophils. Exp Cell Res 154:613-618.

Wang F, Herzmark P, Weiner OD, Srinivasan S, Servant G, and Bourne HR. 2002a. Lipid products of PI(3)Ks maintain persistent cell polarity and directed motility in neutrophils. Nat Cell Biol 4:513-518.

Wang JP, Chang LC, Hsu MF, Chen SC, and Kuo SC. 2002b. Inhibition of formyl-methionyl-leucyl-phenylalanine-stimulated respiratory burst by cirsimaritin involves

inhibition of phospholipase D signaling in rat neutrophils. Naunyn Schmiedebergs Arch Pharmacol 366:307-314.

Wang JP, Chang LC, Hsu MF, and Lin CN. 2003. The blockade of formyl peptide-induced respiratory burst by 2',5'-dihydroxy-2-furfurylchalcone involves phospholipase D signaling in neutrophils. Naunyn Schmiedebergs Arch Pharmacol 368:166-174.

Ward MD and Hammer DA. 1993. A theoretical analysis for the effect of focal contact formation on cell-substrate attachment strength. Biophys J 64:936-959.

Watts RG, Crispens MA, and Howard TH. 1991. A quantitative study of the role of F-actin in producing neutrophil shape. Cell Motil Cytoskeleton 19:159-168.

Watts RG and Howard TH. 1994. Role of tropomyosin, alpha-actinin, and actin binding protein 280 in stabilizing Triton insoluble F-actin in basal and chemotactic factor activated neutrophils. Cell Motil Cytoskeleton 28:155-164.

Waugh MG, Lawson D, and Hsuan JJ. 1999. Epidermal growth factor receptor activation is localized within low-buoyant density, non-caveolar membrane domains. Biochem J 337:591-597.

Waugh RE. 1983. Effects of abnormal cytoskeletal structure on erythrocyte membrane mechanical properties. Cell Motil 3:609-622.

Weernink PA, Guo Y, Zhang C, Schmidt M, Eichel-Streiber C, and Jakobs KH. 2000. Control of cellular phosphatidylinositol 4,5-bisphosphate levels by adhesion signals and rho GTPases in NIH 3T3 fibroblasts involvement of both phosphatidylinositol-4-phosphate 5-kinase and phospholipase C. Eur J Biochem 267:5237-5246.

Weiner OD, Neilsen PO, Prestwich GD, Kirschner MW, Cantley LC, and Bourne HR. 2002. A PtdInsP(3)- and Rho GTPase-mediated positive feedback loop regulates neutrophil polarity. Nat Cell Biol 4:509-513.

Weiner OD, Rentel MC, Ott A, Brown GE, Jedrychowski M, Yaffe MB, Gygi SP, Cantley LC, Bourne HR, and Kirschner MW. 2006. Hem-1 complexes are essential for Rac activation, actin polymerization, and myosin regulation during neutrophil chemotaxis. PLoS Biol 4:e38.

Weiner OD, Servant G, Welch MD, Mitchison TJ, Sedat JW, and Bourne HR. 1999. Spatial control of actin polymerization during neutrophil chemotaxis. Nat Cell Biol 1:75-81.

Weiss-Haljiti C, Pasquali C, Ji H, Gillieron C, Chabert C, Curchod ML, Hirsch E, Ridley AJ, van Huijsduijnen RH, Camps M, and Rommel C. 2004. Involvement of phosphoinositide 3-kinase gamma, Rac, and PAK signaling in chemokine-induced macrophage migration. J Biol Chem 279:43273-43284.

Welch H and Maridonneau-Parini I. 1997. Lyn and Fgr are activated in distinct membrane fractions of human granulocytic cells. Oncogene 15:2021-2029.

Welch HC, Coadwell WJ, Ellson CD, Ferguson GJ, Andrews SR, Erdjument-Bromage H, Tempst P, Hawkins PT, and Stephens LR. 2002. P-Rex1, a PtdIns(3,4,5)P3- and Gbetagamma-regulated guanine-nucleotide exchange factor for Rac. Cell 108:809-821.

Wen Z and Zheng JQ. 2006. Directional guidance of nerve growth cones. Curr Opin Neurobiol 16:52-58.

Wenzel-Seifert K, Hurt CM, and Seifert R. 1998a. High constitutive activity of the human formyl peptide receptor. J Biol Chem 273:24181-24189.

Wenzel-Seifert K, Hurt CM, and Seifert R. 1998b. High constitutive activity of the human formyl peptide receptor. J Biol Chem 273:24181-24189.

Whatmore J, Morgan CP, Cunningham E, Collison KS, Willison KR, and Cockcroft S. 1996. ADP-ribosylation factor 1-regulated phospholipase D activity is localized at the plasma membrane and intracellular organelles in HL60 cells. Biochem J 320 (Pt 3):785-794.

Whimbey E, Gold JW, Polsky B, Dryjanski J, Hawkins C, Blevins A, Brannon P, Kiehn TE, Brown AE, and Armstrong D. 1986. Bacteremia and fungemia in patients with the acquired immunodeficiency syndrome. Ann Intern Med 104:511-514.

Whimbey E, Kiehn TE, Brannon P, Blevins A, and Armstrong D. 1987. Bacteremia and fungemia in patients with neoplastic disease. Am J Med 82:723-730.

Wiebe JP, Kowalik A, Gallardi RL, Egeler O, and Clubb BH. 2000. Glycerol disrupts tight junction-associated actin microfilaments, occludin, and microtubules in Sertoli cells. J Androl 21:625-635.

Williams S, Meij JT, and Panagia V. 1995. Membrane phospholipids and adrenergic receptor function. Mol Cell Biochem 149-150:217-221.

Williamson P, Antia R, and Schlegel RA. 1987. Maintenance of membrane phospholipid asymmetry. Lipid-cytoskeletal interactions or lipid pump? FEBS Lett 219:316-320.

Williamson P and Schlegel RA. 1994. Back and forth: the regulation and function of transbilayer phospholipid movement in eukaryotic cells. Mol Membr Biol 11:199-216.

Wilson MJ, Richter-Lowney K, and Daleke DL. 1993. Hyperglycemia induces a loss of phospholipid asymmetry in human erythrocytes. Biochemistry 32:11302-11310.

Wisniewska A, Draus J, and Subczynski WK. 2003. Is a fluid-mosaic model of biological membranes fully relevant? Studies on lipid organization in model and biological membranes. Cell Mol Biol Lett 8:147-159.

Witke W, Sharpe AH, Hartwig JH, Azuma T, Stossel TP, and Kwiatkowski DJ. 1995. Hemostatic, inflammatory, and fibroblast responses are blunted in mice lacking gelsolin. Cell 81:41-51.

Witko-Sarsat V, Rieu P, Descamps-Latscha B, Lesavre P, and Halbwachs-Mecarelli L. 2000. Neutrophils: molecules, functions and pathophysiological aspects. Lab Invest 80:617-653.

Witt DJ, Craven DE, and McCabe WR. 1987. Bacterial infections in adult patients with the acquired immune deficiency syndrome (AIDS) and AIDS-related complex. Am J Med 82:900-906.

Wollscheid B, von Haller PD, Yi E, Donohoe S, Vaughn K, Keller A, Nesvizhskii AI, Eng J, Li XJ, Goodlett DR, Aebersold R, and Watts JD. 2004. Lipid raft proteins and their identification in T lymphocytes. Subcell Biochem 37:121-152.

Wolven AK, Belmont LD, Mahoney NM, Almo SC, and Drubin DG. 2000. In vivo importance of actin nucleotide exchange catalyzed by profilin. J Cell Biol 150:895-904.

Wong K, Pertz O, Hahn K, and Bourne H. 2006. Neutrophil polarization: spatiotemporal dynamics of RhoA activity support a self-organizing mechanism. Proc Natl Acad Sci U S A 103:3639-3644.

Wu D. 2005. Signaling mechanisms for regulation of chemotaxis. Cell Res 15:52-56.

Wu D, Huang CK, and Jiang H. 2000. Roles of phospholipid signaling in chemoattractant-induced responses. J Cell Sci 113 (Pt 17):2935-2940.

Xian W and Janmey PA. 2002. Dissecting the gelsolin-polyphosphoinositide interaction and engineering of a polyphosphoinositide-sensitive gelsolin C-terminal half protein. J Mol Biol 322:755-771.

Xiao H, Heeringa P, Hu P, Liu Z, Zhao M, Aratani Y, Maeda N, Falk RJ, and Jennette JC. 2002. Antineutrophil cytoplasmic autoantibodies specific for myeloperoxidase cause glomerulonephritis and vasculitis in mice. J Clin Invest 110:955-963.

Xiao H, Heeringa P, Liu Z, Huugen D, Hu P, Maeda N, Falk RJ, and Jennette JC. 2005. The role of neutrophils in the induction of glomerulonephritis by anti-myeloperoxidase antibodies. Am J Pathol 167:39-45.

Xiao RP, Cheng H, Zhou YY, Kuschel M, and Lakatta EG. 1999. Recent advances in cardiac beta(2)-adrenergic signal transduction. Circ Res 85:1092-1100.

Xiao Z and Devreotes PN. 1997. Identification of detergent-resistant plasma membrane microdomains in dictyostelium: enrichment of signal transduction proteins. Mol Biol Cell 8:855-869.

Xie Y, Ding YQ, Hong Y, Feng Z, Navarre S, Xi CX, Zhu XJ, Wang CL, Ackerman SL, Kozlowski D, Mei L, and Xiong WC. 2005. Phosphatidylinositol transfer protein-alpha in netrin-1-induced PLC signalling and neurite outgrowth. Nat Cell Biol 7:1124-1132.

Xu J, Wang F, Van Keymeulen A, Herzmark P, Straight A, Kelly K, Takuwa Y, Sugimoto N, Mitchison T, and Bourne HR. 2003. Divergent signals and cytoskeletal assemblies regulate self-organizing polarity in neutrophils. Cell 114:201-214.

Xu X and London E. 2000. The effect of sterol structure on membrane lipid domains reveals how cholesterol can induce lipid domain formation. Biochemistry 39:843-849.

Yamamori T, Inanami O, Nagahata H, and Kuwabara M. 2004. Phosphoinositide 3-kinase regulates the phosphorylation of NADPH oxidase component p47(phox) by controlling cPKC/PKCdelta but not Akt. Biochem Biophys Res Commun 316:720-730.

Yan SR, Fumagalli L, and Berton G. 1996. Activation of SRC family kinases in human neutrophils. Evidence that p58C-FGR and p53/56LYN redistributed to a Triton X-100-insoluble cytoskeletal fraction, also enriched in the caveolar protein caveolin, display an enhanced kinase activity. FEBS Lett 380:198-203.

Yang C, Huang M, DeBiasio J, Pring M, Joyce M, Miki H, Takenawa T, and Zigmond SH. 2000. Profilin enhances Cdc42-induced nucleation of actin polymerization. J Cell Biol 150:1001-1012.

Yang D, Chen Q, Le Y, Wang JM, and Oppenheim JJ. 2001b. Differential regulation of formyl peptide receptor-like 1 expression during the differentiation of monocytes to dendritic cells and macrophages. J Immunol 166:4092-4098.

Yang D, Chertov O, and Oppenheim JJ. 2001a. The role of mammalian antimicrobial peptides and proteins in awakening of innate host defenses and adaptive immunity. Cell Mol Life Sci 58:978-989.

Yart A, Laffargue M, Mayeux P, Chretien S, Peres C, Tonks N, Roche S, Payrastre B, Chap H, and Raynal P. 2000. A critical role for phosphoinositide 3-kinase upstream of Gab1 and SHP2 in the activation of Ras and mitogen-activated protein kinases by epidermal growth factor. J Biol Chem ..

Yasui K, Becker EL, and Sha'afi RI. 1992. Lipopolysaccharide and serum cause the translocation of G-protein to the membrane and prime neutrophils via CD14. Biochem Biophys Res Commun 183:1280-1286.

Yasui K, Yamazaki M, Miyabayashi M, Tsuno T, and Komiyama A. 1994. Signal transduction pathway in human polymorphonuclear leukocytes for chemotaxis induced by a chemotactic factor. Distinct from the pathway for superoxide anion production. J Immunol 152:5922-5929.

Yonemura S, Nagafuchi A, Sato N, and Tsukita S. 1993. Concentration of an integral membrane protein, CD43 (leukosialin, sialophorin), in the cleavage furrow through the interaction of its cytoplasmic domain with actin-based cytoskeletons. J Cell Biol 120:437-449.

Yoshimori T, Keller P, Roth MG, and Simons K. 1996. Different biosynthetic transport routes to the plasma membrane in BHK and CHO cells. J Cell Biol 133:247-256.

Yu FX, Johnston PA, Sudhof TC, and Yin HL. 1990. gCap39, a calcium ion- and polyphosphoinositide-regulated actin capping protein. Science 250:1413-1415.

Yu S, Kojima N, Hakomori SI, Kudo S, Inoue S, and Inoue Y. 2002. Binding of rainbow trout sperm to egg is mediated by strong carbohydrate-to-carbohydrate interaction between (KDN)GM3 (deaminated neuraminyl ganglioside) and Gg3-like epitope. Proc Natl Acad Sci U S A 99:2854-2859.

Yu W, Cassara J, and Weller PF. 2000. Phosphatidylinositide 3-kinase localizes to cytoplasmic lipid bodies in human polymorphonuclear leukocytes and other myeloid-derived cells. Blood 95:1078-1085.

Yu Y, Chipot C, Cai W, and Shao X. 2006. Molecular Dynamics Study of the Inclusion of Cholesterol into Cyclodextrins. J Phys Chem B Condens Matter Mater Surf Interfaces Biophys 110:6372-6378.

Yusuf HK, Pohlentz G, Schwarzmann G, and Sandhoff K. 1983. Ganglioside biosynthesis in Golgi apparatus of rat liver. Stimulation by phosphatidylglycerol and inhibition by tunicamycin. Eur J Biochem 134:47-54.

Zadeh AD and Keller H. 2003. Chemotactically directed redistribution of alpha-actinin precedes morphological polarization and reversal of polarity in human polymorphonuclear leucocytes (PMNs). Eur J Cell Biol 82:93-96.

Zarkowsky HS, Mohandas N, Speaker CB, and Shohet SB. 1975. A congenital haemolytic anaemia with thermal sensitivity of the erythrocyte membrane. Br J Haematol 29:537-543.

Zegers MM and Hoekstra D. 1998. Mechanisms and functional features of polarized membrane traffic in epithelial and hepatic cells. Biochem J 336:2-69.

Zegers MM, Zaal KJ, van IJzendoorn SC, Klappe K, and Hoekstra D. 1998. Actin filaments and microtubules are involved in different membrane traffic pathways that

transport sphingolipids to the apical surface of polarized HepG2 cells. Mol Biol Cell 9:1939-1949.

Zhang XL, Topley N, Ito T, and Phillips A. 2005. Interleukin-6 regulation of transforming growth factor (TGF)-beta receptor compartmentalization and turnover enhances TGF-beta1 signaling. J Biol Chem 280:12239-12245.

Zheng L, Eckerdal J, Dimitrijevic I, and Andersson T. 1997. Chemotactic peptide-induced activation of Ras in human neutrophils is associated with inhibition of p120-GAP activity. J Biol Chem 272:23448-23454.

Zheng L, Shan J, Krishnamoorthi R, and Wang X. 2002. Activation of plant phospholipase Dbeta by phosphatidylinositol 4,5-bisphosphate: characterization of binding site and mode of action. Biochemistry 41:4546-4553.

Zigmond SH. 1974. Mechanisms of sensing chemical gradients by polymorphonuclear leukocytes. Nature 249:450-452.

Zigmond SH. 1977. Ability of polymorphonuclear leukocytes to orient in gradients of chemotactic factors. J Cell Biol 75:606-616.

Zigmond SH, Levitsky HI, and Kreel BJ. 1981. Cell polarity: an examination of its behavioral expression and its consequences for polymorphonuclear leukocyte chemotaxis. J Cell Biol 89:585-592.

Zimerman B, Volberg T, and Geiger B. 2004. Early molecular events in the assembly of the focal adhesion-stress fiber complex during fibroblast spreading. Cell Motil Cytoskeleton 58:143-159.

Zimmerberg J, Vogel SS, and Chernomordik LV. 1993. Mechanisms of membrane fusion. Annu Rev Biophys Biomol Struct 22:433-466.

Zwaal RF and Bevers EM. 1983. Platelet phospholipid asymmetry and its significance in hemostasis. Subcell Biochem 9:299-334.

CHAPTER 2

PREPARATION OF SECRETORY VESICLE-FREE PLASMA MEMBRANES BY ISOPYCNIC SUCROSE GRADIENT FRACTIONATION OF NEUTROPHILS PURIFIED BY THE GELATIN METHOD

Abstract

Isolated human neutrophils serve as a model for the in-vitro study of host defensive processes as well as the cell biology and biochemistry of primary human cells. We demonstrate that the requirements of the gelatin-based procedure for neutrophil isolation from whole blood induces the complete loss of secretory vesicles from in-vitro isolated populations, whereas isolation by a dextran-based methodology results in the preservation of this organelle. Following density fractionation of cellular cavitates, examination of commonly employed plasma membrane marker activities yielded subcellular localization patterns that were indistinguishable between dextran- or gelatin-isolated populations, indicating both populations to be otherwise comparable in terms of the relative complexity and large-scale organization of plasma membranes. Given that the cell surface upregulation of secretory vesicles is implicated as an initial requirement of neutrophil activation as well as an intrinsic feature of neutrophil priming, we show that dextran- and gelatin-isolated neutrophils may be considered to occupy functionally nonactivated and primed cellular states, respectively. These differences in phenotype can be exploited in specific ways. We suggest that the gelatin method has technical advantages with regard to the study of neutrophil plasma membranes. In particular,

results from this study indicate the gelatin method to be a reliable and effective preparatory technique appropriate for tandem use with density fractionation procedures to achieve rapid isolation of plasma membranes that are uncontaminated by secretory organelles.

Abbreviations

AP, alkaline phosphatase; AMP-5' NT, adenosine mono-phosphate-dependent 5' nucleotidase; HLA, human leukocyte antigen; LF, lactoferrin; Mg^{++} ATPase, magnesium-dependent adenosine tri-phosphatase; MPO, myeloperoxidase

Introduction

Neutrophils are widely recognized as the principle effectors of the innate immune response. The rapid recruitment and activation of circulating, quiescent leukocytes poses a unique battery of cellular, physiological and pathophysiological requirements on responding effector granulocytes, including the secure sequestration, maintenance, and regulated release of preformed microbiocidal proteins and peptides. Circulating, quiescent neutrophils house three distinct granule subsets, including primary (azurophil), secondary (specific) and tertiary (gelatinase-enriched) granules, as well as a stable endosomal subpopulation referred to as secretory vesicles (Borregaard and Cowland, 1997). The mobilization of granules and secretory vesicles is differentially regulated, with the cell surface upregulation of each subset governed independently by specific thresholds of cellular activation. Pattern of release is, from the most-to-least readily

mobilized, secretory vesicles > tertiary > secondary > primary granules (Sengelov et al., 1993). Selective and sequential mobilization confers progressive changes in cellular phenotype that underlie hallmark features of neutrophil defensive function in vivo (Faurschou and Borregaard, 2003). Initial cell surface upregulation of secretory vesicles is required for neutrophil extravasation and is thus implicated as a general condition of neutrophil activation (Sengelov et al., 1995;Borregaard et al., 1994).

Secretory vesicles are plasma membrane-derived organelles formed during the latter stages of neutrophil development (Borregaard, 1997). As would be expected, the physical properties of this organelle substantially overlap those of surface membranes and prevent purification of either fraction on basis of relative density (Borregaard et al., 1990). Detailed studies examining the biology of secretory vesicle dynamics within human neutrophils suggest that this organelle is inherently unstable within in-vitro isolated cells and readily lost following exposure to various environmental (Borregaard et al., 1987) and/or physiologic factors (Borregaard et al., 1994). Thus in many fields of neutrophil research that require extensive in-vitro manipulation of isolated populations, including the study of priming (Condliffe et al., 1998), secretory vesicles are widely assumed to be uniformly upregulated to the cell surface of intact cells under study. However, this assumption, although convenient and justifiable, may not be as absolute as currently considered. For example, high-resolution electron microscopy techniques based on combined immuno- and enzyme-cytochemistry have been applied to visualize the dynamic behavior secretory vesicles within intact fMLF-stimulated human neutrophil populations and indicate the mobilization of this organelle to surface membranes may be

more intricate and complex than is generally appreciated (Kobayashi and Robinson, 1991). Other studies indicate cell surface mobilization kinetics of secretory vesicles can be substantially altered in a stimulus-specific manner (Ward et al., 2000). These studies suggest an unexpected complexity and variability associated with secretory organelle loss and underscore the importance of accounting for secretory vesicle loss in various experimental systems. Thus, a detailed accounting of the fate of this organelle following neutrophil isolation by a common gelatin-based procedure (Henson and Oades, 1975), which is known to promote an intermediate stage of neutrophil activation, is appropriate. Findings in this regard could provide insight into the physiologic changes induced in neutrophils isolated by this method and may, as a result, additionally contribute to the clarification of previous controversial findings in neutrophil research.

In this study we compare the differential dynamics of secretory vesicle mobilization induced following neutrophil isolation using two preparative methods that differ substantially. Procedures employing either gelatin or dextran to achieve primary separation of erythrocytes from leukocytes were used to isolate neutrophil populations from whole blood. Each method has distinct advantages that allow isolation of neutrophil populations in very different, if not reciprocal, cellular activation states. In particular, the dextran-based approach is a relatively gentle method that largely preserves secretory organelles within isolated populations (Borregaard et al., 1990). Thus, neutrophils isolated by the dextran method may be considered to occupy a nonactivated or unprimed cellular state and were useful in this study as a control for examining the extent of secretory vesicle upregulation and the uniformity of their integration in surface

membranes. In contrast, the conditions imposed by the gelatin procedure should promote LPS- as well as temperature-dependent changes in neutrophil physiology that could, in turn, induce loss of the secretory compartment in isolated populations. We demonstrate that the methodological requirements of the gelatin protocol are indeed sufficient to induce complete mobilization of secretory vesicles as well as their uniform integration into existing cell surface structure. Such populations may be considered operationally primed based on the complete absence of secretory vesicles (Faurschou and Borregaard, 2003;Borregaard et al., 1994). An additional novel aspect of this study includes the subcellular characterization of intact secretory vesicles relative to plasma membranes and major granule subsets by isopycnic sucrose density gradient sedimentation.

Materials and Methods

Neutrophil Isolation

The dextran method of neutrophil isolation is performed essentially as described by Borregaard et al. (Borregaard et al., 1983) with minor modification. Whole blood is gently mixed in a 1:1 volumetric ratio with 150 mM NaCl solution containing 3% dextran made with WFI-grade distilled water within an autoclaved 1L separatory funnel. After 30-to-45 minutes at room temperature, the resulting suspension is centrifuged (740 xg, 10 min, brake off, 25˚C chamber) and pellets are resuspended in several milliliters of room temperature 150 mM NaCl solution and transferred into sterile 50 ml conical tubes. Additional saline (25˚C) is added to a final volume of 35 ml, and the mixture underlaid with 10 ml Ficoll-Hypaque medium for gradient sedimentation (415 xg, 25 min, brake

off, 25˚C chamber). Neutrophil-rich pellets are resuspended in 25 ml ice-cold distilled water (WFI-grade) and agitated by repeated up/down pipetting for 20-to-25 seconds to facilitate lysis of residual red cells. Isotonicity is restored by addition of an equal volume of ice-cold 300 mM NaCl solution. One-to-two cycles are required to fully eliminate red cells. Cells are then sedimented (415 xg, 10 min, brake on, 4˚C chamber) and pellets finally resuspended in 25 ml $DPBS^{4+}$. Suspensions are transferred to ice for 15 minute treatment with 3 mM DFP. Prior to DFP addition, we remove a small aliquot of cells for cell count determination by hemocytometer. 3 mM DFP is then applied followed by 15-minute incubation period on ice. DFP-treated suspensions are sedimented (415 xg, 10 min, brake on, 4˚C chamber), washed 1-to-2 times in the same buffer, and either resuspended in 10ml $DPBS^{4+}$ for radiolabeling (see below) or 5 ml cavitation buffer (0.34 M sucrose, 10 mM HEPES, pH 7.4, 1 mM EDTA, 0.1 mM MgCl2, 1 mM ATP, 0.1 mM PMSF, pH 7.4, and protease inhibitor cocktail, added as recommended by manufacturer). We employ N_2 cavitation for cell disruption at 450 psi for 15 minutes at 4˚C. Nuclei and cellular debris are removed from cavitates by low speed sedimentation (890 xg, 10 min, brake on, 4˚C chamber). In some cases, we additionally extract nuclear pellets in an iced Dounce homogenizer with 5 strokes of a type A pestle; the nuclear extract is re-sedimented as above and the soluble fraction added to the appropriate low speed supernatant. Low speed supernatants from each population are separately pooled into a final volume of 5 ml and layered on top of an already prepared linear sucrose gradient (see below).

The gelatin method of neutrophil isolation is performed essentially as described by Henson and Oades (Henson and Oades, 1975) with minor modification. One unit of donor blood is transferred into two 250 ml sedimentation tubes and sedimented at low speed (260 xg, 20 min, brake off, 25˚C chamber) to separate plasma and non-granular leukocytes from PMN/red cell-enriched pellets. PMN/red cell-enriched pellets are then transferred into a sterile 1L separatory funnel. 500 ml (per unit blood) gelatin solution (2% gelatin in 150 mM NaCl), pre-warmed to 37˚C, is then added, some of which is used to recover remaining cells from sedimentation tubes. The separatory funnel is tightly sealed, rotated gently end-over-end, positioned upright with the aid of funnel holder, and transferred to a 37˚C incubator. After 30 minutes incubation, the separatory funnel is removed from the incubation chamber, the lid is loosened, and red cell pellets are removed through a stopcock fitted at the lower end. The upper phase of the suspension is transferred into two 250 ml sedimentation tubes, centrifuged (360 xg, 10 min, brake on, 25˚C chamber), and the cell pellets obtained are resuspended directly into 75 ml ice-cold ammonium chloride lysis buffer (155 mM NH_4Cl, 9.4 mM $NaHCO_3$, pH 7.4, and 130 μM EDTA). Resuspended pellets are then pooled, transferred to ice and incubated 5 minutes with continuous agitation produced by repeated up/down pipetting of the mixture. Treated suspensions are sedimented (360 xg, 10 min, brake on, 4˚C chamber), washed 1-2 times in 25 ml $DPBS^{4+}$, and finally transferred to a sterile 50 ml conical tube for DFP treatment in the same buffer (see above). Cell counts, cell lysis, and preparation of low speed supernatants are as described above. It is essential that plastic-ware directly

contacting blood during the course of gelatin isolation is not re-used during consecutive steps of this procedure (see Results and Discussion Section).

Isopycnic Sucrose Gradient Sedimentation

Sucrose solutions are made with 10 mM HEPES, pH 7.4. Linear gradients are formed using a gradient maker[10] with 11 ml 20% (w/w) and 11 ml 55% (w/w) sucrose solutions in the flow and mixing columns, respectively. Prior to pouring gradients, a 3 ml cushion of 60% (w/w) sucrose is added to the sedimentation tube on the receiving end. A peristaltic pump is employed to insure smooth, continuous flow during gradient formation. Gradients are formed at 4˚C and maintained at this temperature for 16-24 hours prior to use. Low speed supernatants are layered on top of gradients and sedimentation tubes transferred into a Beckman[2] SW28 rotor and sedimented 3.5 hours (8.3×10^4 xg, 3.5 hr, 4˚C chamber). We then employ an Auto Densi-Flow IIC fraction collector[9] at 4˚C to fractionate gradients. Fractions can be stored at -70˚C immediately following fractionation or maintained at 4˚C for immediate analysis. Fractions stored at -70˚C must be rapidly thawed in the presence of 0.1 mM PMSF and protease inhibitor cocktail, added as recommended by manufacturer, to prevent proteolysis, and subsequently equilibrated on ice prior to analysis.

Cell Surface Labeling

Iodine-125-conjugated wheat germ agglutinin [^{125}I]WGA was prepared as described previously (Jesaitis et al., 1983). Briefly, cells are suspended in 10 ml $DPBS^{4+}$ and radiolabeled using 70 µg WGA per $5x10^8$ cells with a total applied cpm of $2.5x10^3$

per population. After 5 minutes on ice, cells are washed twice in the same buffer, cavitated, and fractionated as described above. We assessed the [^{125}I] WGA content of gradient fractions directly using a gamma counter (10/200 Plus[12]). The concentration of active [^{125}I]WGA applied per population in this study ($1x10^4$ cpm) represents the minimal amount to obtain acceptable signal/noise ratio and is well below previously reported levels applied without altering normal subcellular distribution pattern of plasma membranes (Quinn et al., 1989;Jesaitis et al., 1982).

Biochemical Assays

Protein measurements are made using the BCA assay kit with BSA as a protein standard. The sucrose density of gradient fractions is calculated by refractive index measurement.

Costar flat-bottom microtiter plates are used for the following biochemical assays**.** Mg^{++} ATPase activity is determined by a modified approach based on two previously described methods (Henkel et al., 1988;Jesaitis et al., 1982). A 10 µl sample volume is added to 40 µl reaction buffer (810 mM histidine, 5.4 M NaCl, 270 mM KCl, and 135 mM $MgCl_2$) and assayed in the presence or absence of 5 mM ATP for 22 minutes at 37˚C. Termination is by addition of 200ul per well of a 1:1:2:2 ratio of 5.72% ammonium molybdate, w/v, in 6 M HCl, 2.32% (w/v) polyvinyl alcohol, 0.0812% (w/v) malachite green, and distilled water, respectively. Reaction initiation/termination is staggered at 10 second intervals to permit synchronization. Terminated samples are allowed to stabilize 5 minutes at room temperature prior to colorimetric quantitation of free phosphate (630 nm). Measurements are acquired using a microtiter plate reader **(**SpectraMax 250).[14] The

amount of inorganic phosphate released is calculated based on analysis of a standard series of phosphate solutions with linear range of detection between 1 and 10 nanomoles phosphate.

AMP-5' NT activity is assayed as described by Emmelot and Bos (Emmelot and Bos, 1966) but modified to allow timed initiation of 50 µl reactions by final addition of 11 mM AMP. Synchronization, reaction termination, and phosphate quantitation are as described above.

MPO activity is directly measured from 40 µl sample volumes following addition of a reaction buffer (50 mM citric acid, pH 4.2, 83.3 µM hydrogen peroxide, 0.0275% ABTS) by microtiter plate reader[14] at wavelength 405 nm.

AP activity is assayed using p-nitrophenylphosphate as substrate (DeChatelet and Cooper, 1970) in the presence or absence of 0.1% Triton X-100. 20 µl samples are added to microtiter plate wells containing 30 µl of a reaction buffer consisting of 0.1 M diethanolamine-HCl buffer, pH 9.75, with 0.33 mM $MgCl_2$. Reactions are initiated by adding 50 µl of 8 mM p-nitrophenylphosphate solution, prepared in the same buffer, and transferred to a 37˚C incubator. Reactions are terminated after 20 minutes at 37˚C by addition of 50 µl 2 M NaOH and analyzed at wavelength 405 nm by microtiter plate reader.[14]

ELISA Detection Assays

Maxisorp immuno-plates are used for ELISAs. HLA activity is assayed by sandwich ELISA according to the method of Bjerrum and Borregaard

(Bjerrum and Borregaard, 1990) using affinity purified sheep anti-human β_2 microglobulin and mouse anti-human HLA class I as capture and detection antibodies, respectively.

For the CD16 assay, immuno-plate wells were coated with 45 µl sample volume for 16-32 hours at 4˚C. Because the predominant cellular fraction of this protein is housed within intact secretory vesicles, individual gradient fractions are freeze-thawed according the method of Philips et al. (Philips et al., 1991) in the presence of 100 µM PMSF and protease inhibitor cocktail, added as recommended by manufacturer, prior to initial coating. Coated wells are continuously rinsed for 5 minutes in wash buffer containing 150 mM NaCl and 10 mM HEPES, pH 7.4, and then exposed to 2 µg CD16 monoclonal antibody, diluted in wash buffer supplemented with 0.5% BSA, for 60 minutes at room temperature with agitation. Plate wells are washed as described above, and a 1:2000 dilution of HRP-conjugated secondary antibody, diluted as above, is applied for 60 minutes, followed by continuous washing. Reaction buffer (50 mM citrate, pH 4.2, 83.3 µM hydrogen peroxide, 0.0275% ABTS) is added and signal quantified spectrophotometrically at wavelength 405 nm. Additional controls applying primary and secondary antibodies to buffer-coated wells are employed to measure associated background noise, which is then subtracted from initial activity readings. Parallel controls run without primary and secondary antibody demonstrate that the HRP-substrate solution is color-stable under these assay conditions. The efficacy of granule lysis by the freeze-thaw method employed (Philips et al., 1991) is evaluated by measuring the relative increase in detergent-free AP activity (see above) before and after treatment of gradient

fractions from dextran isolated neutrophil populations. When compared to AP activity released from control fractions assayed in parallel in the presence of 0.1% Triton-X-100, vesicle disruption by this method is found to be ~97% efficient. A detergent-free approach to vesicle disruption is preferred because inclusion of detergent during the initial coating of immuno-plate wells significantly reduces sensitivity of antigen-detection.

Electrophoresis and Immunoblotting

Gradient fractions are solubilized and denatured in 6x SDS sample buffer in the presence of 0.1 mM PMSF and protease inhibitor cocktail, added as recommended by manufacturer, by transferring lightly vortexed samples to a heating block, pre-warmed to 90˚C, for 5 minutes. Samples are again vortexed, allowed to cool to room temperature, and loaded on 10% polyacrylamide slab gels. The electrophoretic mobility of gradient samples is compared to that of pre-stained molecular weight standard proteins. Protein from gels is electrotransferred onto PVDF membranes and treated as described below.

Immunoblotting is performed at 22˚C as previously described (Jesaitis et al., 1988). Following electrotransfer, PVDF membranes are incubated in blocking solution (0.5 M NaCl, 10 mM HEPES, pH 7.4, 3% BSA, 10% goat serum) for 16-to-32 hours at 4˚C and exposed to 0.5 μg of rabbit polyclonal anti-LF antibody, diluted in DPBS with 3% goat serum, 1% BSA and 0.2% Tween 20, for 60 minutes (25˚C), rinsed five times in wash buffer (0.25M NaCl, 10 mM HEPES, pH 7.4, 0.2% Tween 20) for a total of 25 minutes, and blotted with a 1:20,000 dilution of HRP-conjugated secondary antibody in the same buffer used for the primary antibody. After rinsing membranes as above, signal

is measured using Supersignal chemiluminescent detection kit. Signal is then quantified from optical scans of exposed film using ImagQuant software (Version 3.3).[1]

Results and discussion

Relative differences in cell surface topography can significantly alter the otherwise fundamental biochemical and biophysical properties generally attributed to mammalian cell plasma membranes (DePierre and Karnovsky, 1973). Similarly, surface membrane irregularities resulting from incomplete cell surface integration of various organelle subsets following their mobilization to plasma membranes may be indicated by global alterations in peak sedimentation density of plasma membrane-specific marker activities (Mukherjee et al., 1994). We sought therefore to verify the compositional and structural integrity of plasma membranes from gelatin-isolated neutrophil populations, which may be regarded as LPS-primed (Guthrie et al., 1984;Haslett et al., 1985), by analyzing the subcellular distribution profile of a panel of commonly employed plasma membrane-specific marker activities.

Reference Marker Activities

Experiments examining gelatin- and dextran-isolated neutrophil populations were performed in parallel under identical conditions of cavitation, sedimentation and fractionation. Because sucrose content of gradient fractions rose linearly from 11 to 60% (wt/wt; data not shown), marker activities are expressed in terms of percent sucrose. Comparative analysis of subcellular protein distribution obtained from density fractionated cavitates of gelatin and dextran isolated neutrophils were indistinguishable

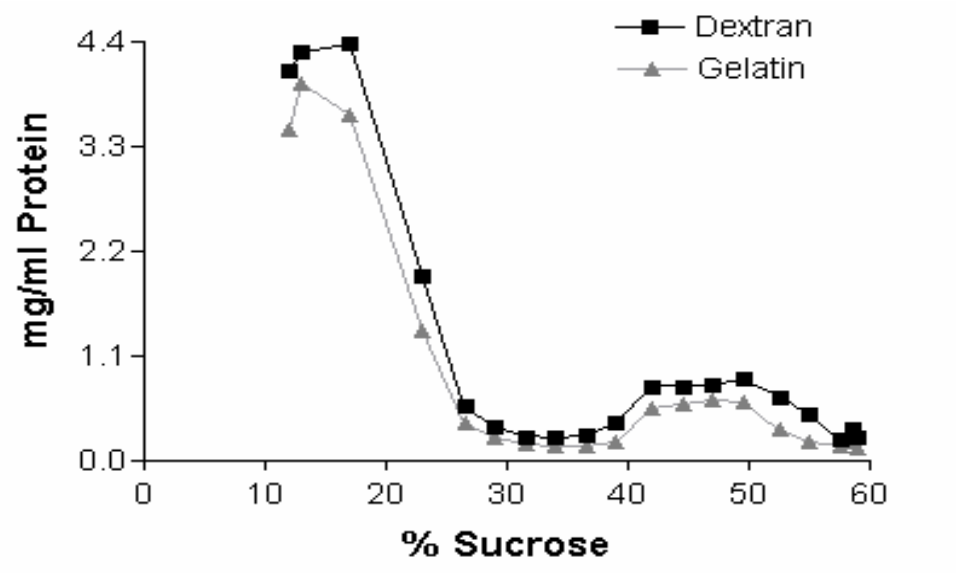
and demonstrate the majority of cellular protein localized within cytosol with minor peaks corresponding to the subcellular location of primary and secondary granules (Fig 1a). Reference markers, LF and MPO, were employed to identify gradient regions enriched in secondary and primary granules, respectively (Fig 1b). As shown in Figure 1b, the subcellular distribution of granule marker activities closely corresponded in gradients formed from either gelatin- or dextran-isolated neutrophil populations.

Plasma Membrane Marker Activities

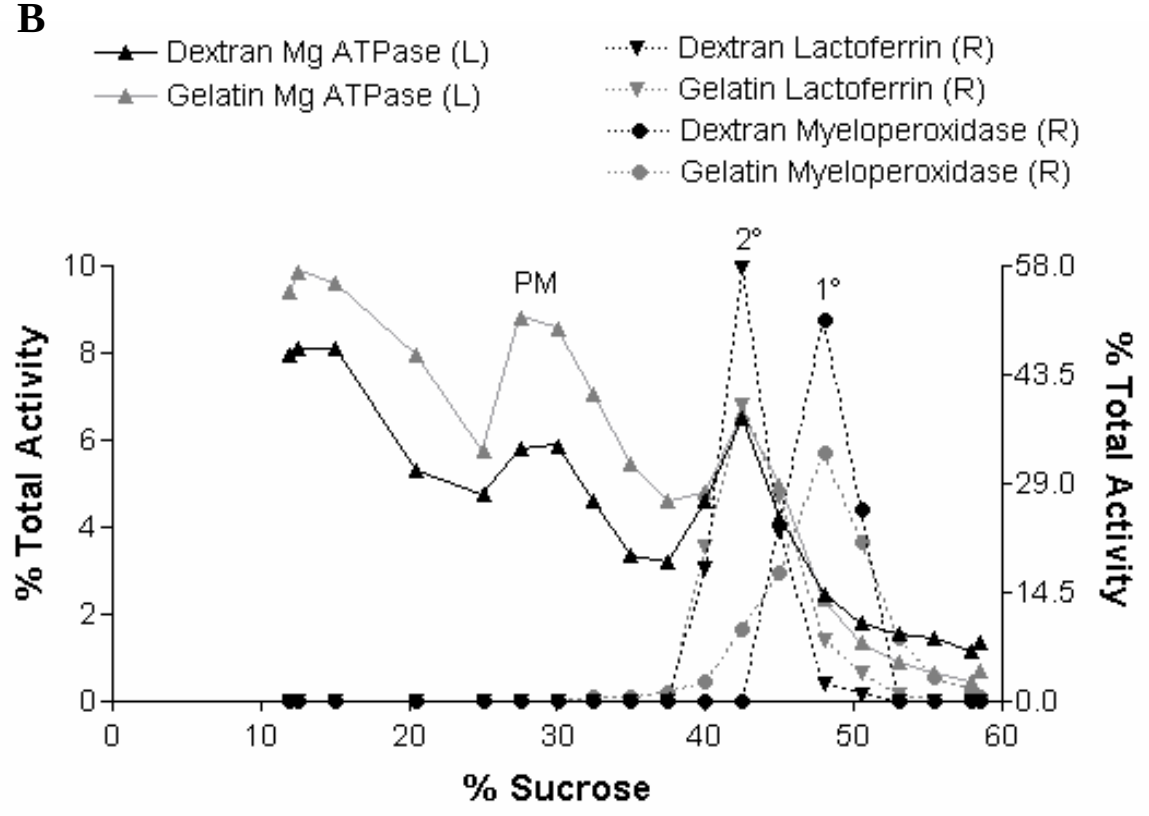
We initially examined Mg^{++} ATPase activity (Harlan et al., 1977) to determine the distribution of plasma membrane-enriched vesicles following density fractionation of cellular cavitates. Figure 1b shows the typical bimodal localization pattern of this activity clearly indicating preferential enrichment within plasma membranes as well as specific granules and is consistent with previous work (Jesaitis et al., 1982). A small shift in cellular Mg^{++} ATPase activity from specific granules to plasma membrane fractions in gelatin-prepared cells, relative to dextran-prepared cells, was consistently observed, suggesting an additional basis of phenotypic distinction between gelatin- and dextran-isolated populations (see below).

Analysis of HLA, AMP-5' NT, and detergent-free AP activity yielded localization patterns that were superimposable between gradients made from either dextran- or gelatin-isolated neutrophils (Fig 1c). Furthermore, these profiles closely correspond with those of Mg^{++} ATPase obtained from gelatin and dextran populations in the ~30% range of sucrose densities (compare Figures 1b and 1c). These data suggest that the physical properties of bulk phase plasma membranes derived from gelatin-isolated neutrophil

A


Dextran
Gelatin
mg/ml Protein
4.4
3.3
2.2
1.1
0.0
0
10
20
30
40
50
60
% Sucrose

B


Dextran Mg ATPase (L)
Gelatin Mg ATPase (L)
Dextran Lactoferrin (R)
Gelatin Lactoferrin (R)
Dextran Myeloperoxidase (R)
Gelatin Myeloperoxidase (R)
PM
2°
1°
% Total Activity
10
8
6
4
2
0
58.0
43.5
29.0
14.5
0.0
0
10
20
30
40
50
60
% Sucrose

C

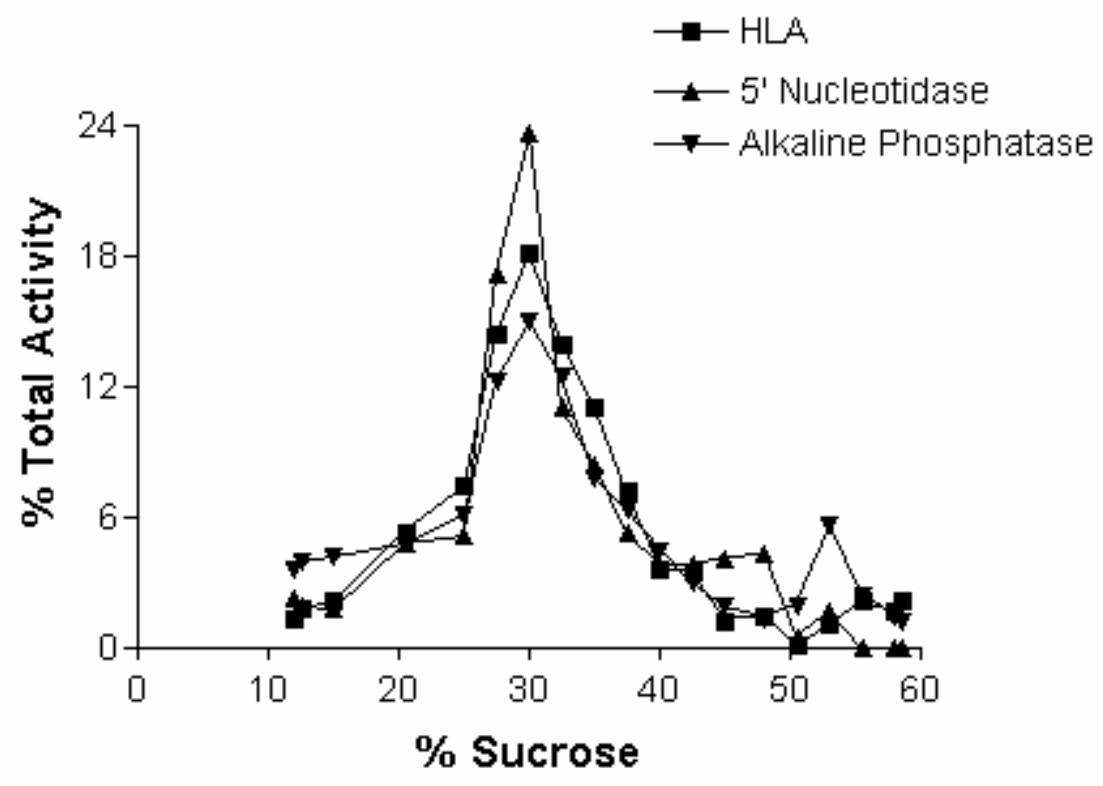


Figure 1. Subcellular distribution of protein, Mg^{++} ATPase, LF and MPO from neutrophil populations prepared by dextran- (black lines) and gelatin- based (gray lines) procedures. A) Subcellular distribution of protein in fractions obtained from cavitates of dextran-prepared cells (■) and gelatin-prepared cells (▲). Protein content of gradient fractions is plotted in units of mg/ml as a function of percent sucrose. Protein recovery from gradient fractions ranged from 89-95%. B) Subcellular distribution of plasma membrane-enriched vesicles was identified by measuring Mg^{++} ATPase activity in fractions prepared from dextran (▲) and gelatin (▲) prepared cells. PM indicates plasma membrane. As indicated, Mg^{++} ATPase is additionally enriched in specific granules. LF content of gradient fractions from dextran (··▼··) and gelatin (··▼··) isolated populations was measured by immuno-blot analysis and used to confirm the subcellular distribution of specific granules, while that of azurophil granules (dextran·· ●··; gelatin ··●··) was identified by measurement of MPO activity, assessed by enzymatic analysis. Marker recovery from gradient fractions exceeded 90%. Data are representative of comparable experiments from 8 gradients, four dextran and four gelatin. Right and left ordinate axes are indicated by 'R' and 'L,' respectively. C) Subcellular distribution of HLA (■), AMP-5' NT (▲), and detergent-free alkaline phosphatase (▼) activities, indicating plasma membrane enrichment. The data shown are representative of similar results obtained from six gradients: three from dextran- and three from gelatin-isolated

(Figure 1 –continued) neutrophil populations. Marker recovery from gradient fractions was ≥84% of the applied activity.

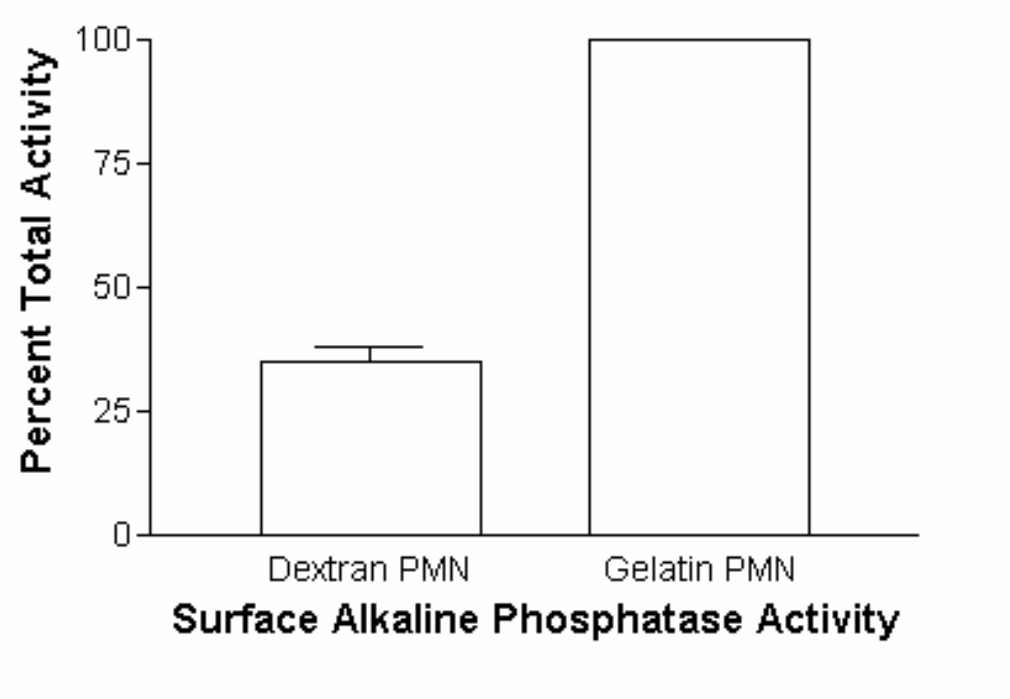
populations are not significantly different as a result of possible complexities associated with cell-surface upregulation of secretory vesicles or previously observed perturbing effects that can potentially result from LPS-plasma membrane interactions (see below).

AP Activity

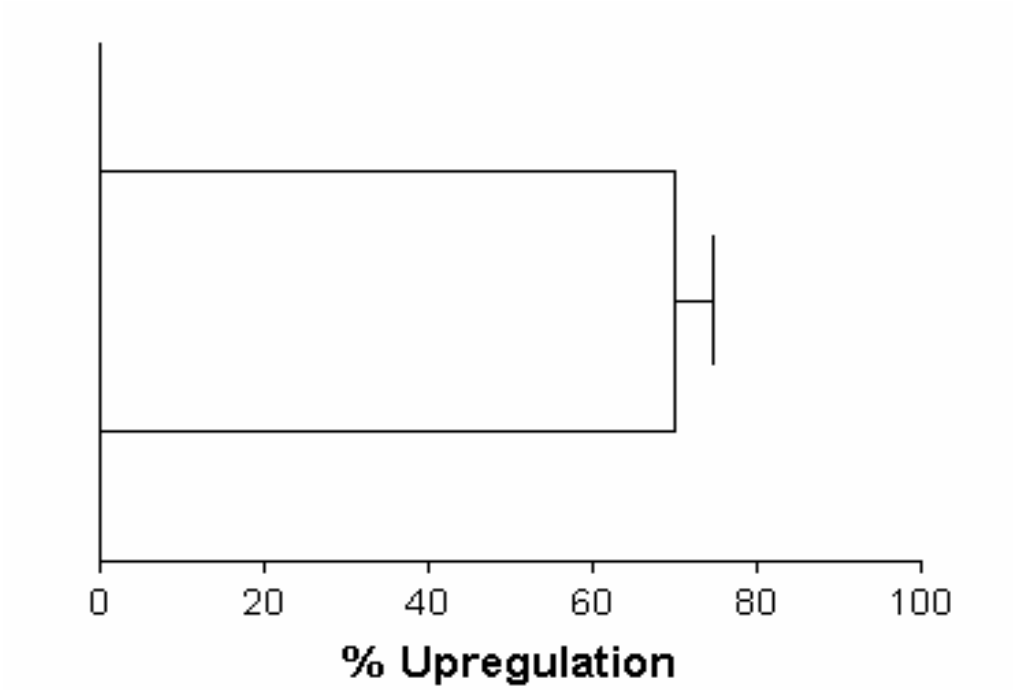
AP is a glycophosphatidylinositol (GPI)-linked protein that is enriched in secretory vesicles. Quantitative measurement of this marker activity in the presence or absence of detergent can therefore serve as a primary indicator for extent of secretory vesicle loss within in-vitro isolated neutrophil populations (Borregaard et al., 1990). Following density fractionation of cellular cavitates, dextran-isolated neutrophils were found to retain from 60 to 70% of their secretory vesicle complement (Fig 2a). Gelatin isolated neutrophils, however, completely lacked this organelle (Figs 2a), as AP activity showed no sensitivity to detergent in these populations. The absence of error bars for the gelatin-isolated populations analyzed in figure 2a is due to the lack of detectable latent AP activity. These results are highly reproducible but only when certain precautions are observed.

More specifically, we have found that re-use of various plastics, including sedimentation tubes, pipettes, and seperatory funnels, throughout the gelatin-isolation procedure promotes from 20-to-40% retention of secretory vesicles within neutrophil populations (Fig 2b). It is possible that the repeated exposure of cells to plasma-coated surfaces modulates cellular activation potential during the course of isolation. This

A


Percent Total Activity
100
75
50
25
0
Dextran PMN
Gelatin PMN
Surface Alkaline Phosphatase Activity

B


0
20
40
60
80
100
% Upregulation

C

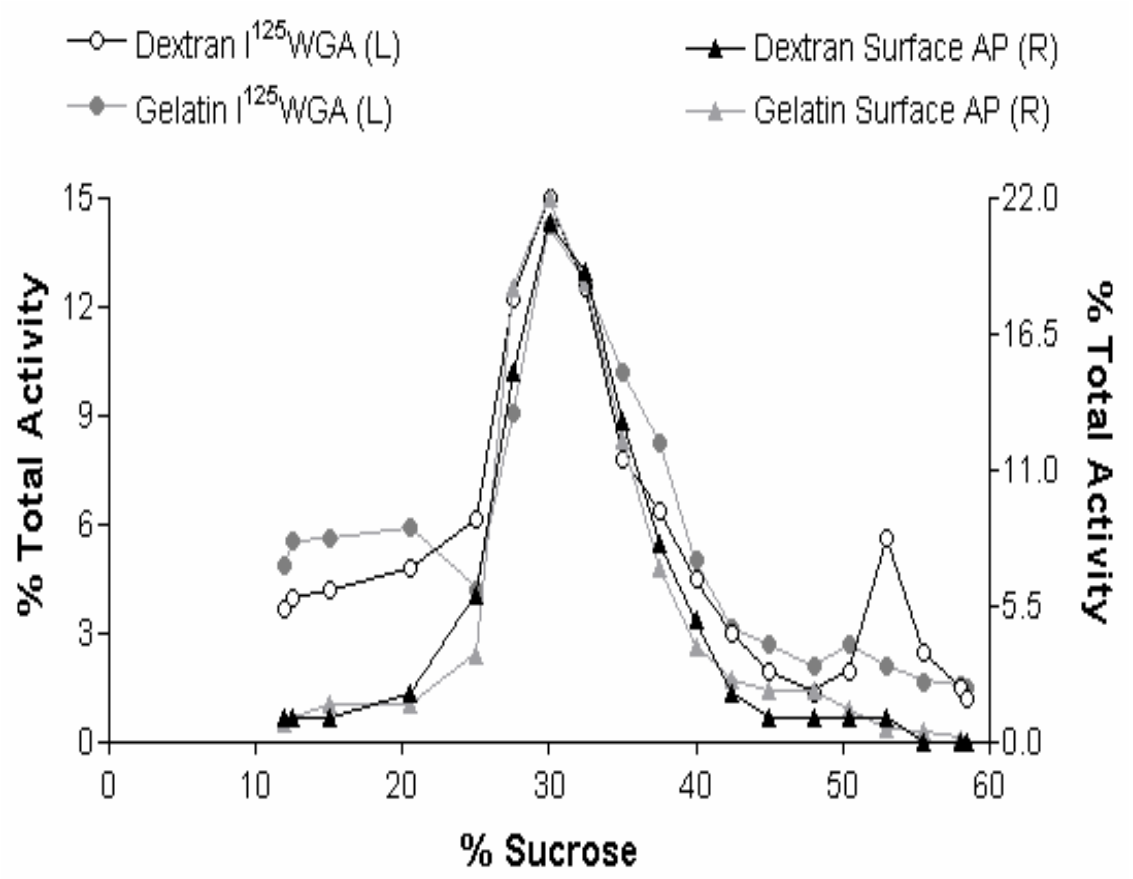


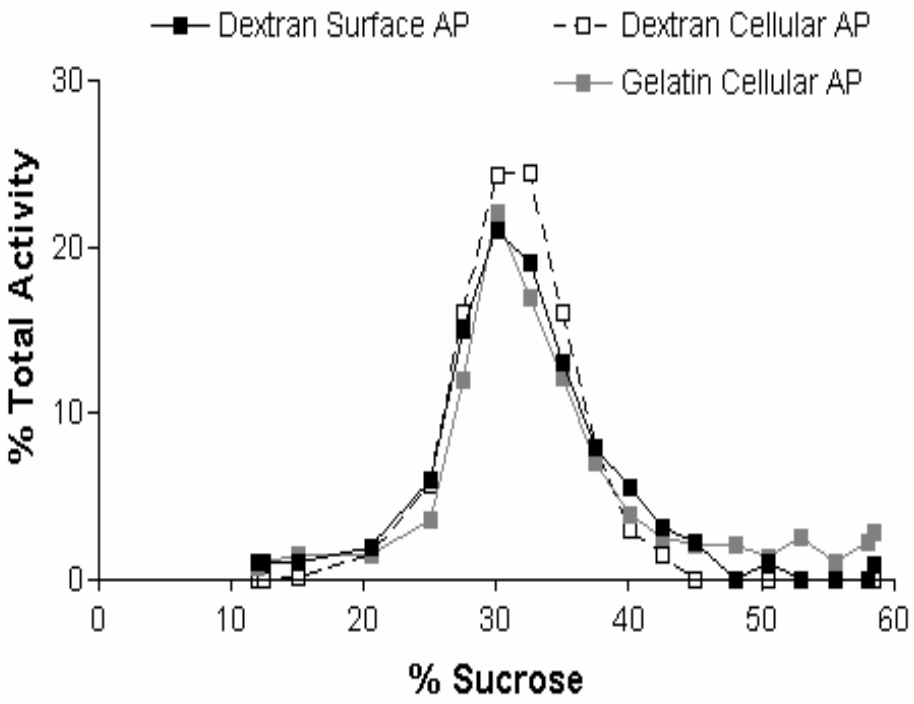
Figure 2. Distribution of cellular alkaline phosphatase activity from dextran and gelatin isolated neutrophil populations and subcellular localization of latent activity in density fractionated lysates. A) Gradient fractions were assayed for alkaline phosphatase activity in the absence and presence of 0.1% Triton-X-100 to determine relative surface-localized and total cellular activity, respectively. Data obtained from analysis of eight gradients, four dextran and four gelatin, are shown. B) Percent of cellular AP activity that is associated with the cell surface following gelatin-isolation with re-use of various plastic-ware during the procedure (see text). Data are from four gradients, each made with neutrophil populations obtained from different donors. C) Comparison of surface-associated alkaline phosphatase activity from dextran (▲) and gelatin (▲) prepared cells with subcellular distribution of cell surface label, [^{125}I]WGA, in dextran (○) and gelatin (●) gradients. Right and left ordinate axes are indicated by 'R' and 'L,' respectively. Recovery of [^{125}I]WGA from gradient fractions ranged from 85-93%. Data are representative of comparable experiments from 8 gradients, including four dextran and

(Figure 2 –continued) four gelatin. Error bars show standard error of the mean. Recovery of alkaline phosphatase activity from gradient fractions exceeded 90%.

explanation is consistent with the immunoregulatory effects of certain plasma components on neutrophil behavior described in previous studies (Balke et al., 1995;Tschesche et al., 1994) as well as our own observations of complete secretory organelle loss being contingent on the use of clean, fresh materials during each isolation step. The application of new or clean plastic-ware during neutrophil isolation by the gelatin protocol is therefore essential to the successful reproduction of results described in this study.

The incubation requirements for gelatin-mediated red cell sedimentation in the presence of LPS very likely underlie the complete surface upregulation of secretory vesicles in isolated populations. Accordingly, exposure of neutrophils to as little as 50 pg LPS for 30 minutes at 37˚C induces notable and multiple priming effects on treated populations (Dahinden et al., 1983) and is consistent with the complete upregulation of secretory vesicles in the populations under study (Borregaard et al., 1994). Alternatively, direct neutrophil-LPS interactions occurring during the course of 37˚C incubation may substantially delay (Ward et al., 2000) the otherwise rapid temperature-dependent mobilization of secretory vesicles (Borregaard et al., 1987). These latter observations are consistent with results obtained from high-resolution single cell studies, which suggest that intracellular behavior of this organelle may affect their differential and incomplete integration within plasma membrane structure (Kobayashi and Robinson, 1991). In addition, previously observed nonspecific unsaturable adsorption of LPS by plasma membranes (Wilson et al., 1982) may also contribute to irregularities in cell surface

A


Dextran Surface AP
Dextran Cellular AP
Gelatin Cellular AP
% Total Activity
% Sucrose
0
10
20
30
40
50
60

B

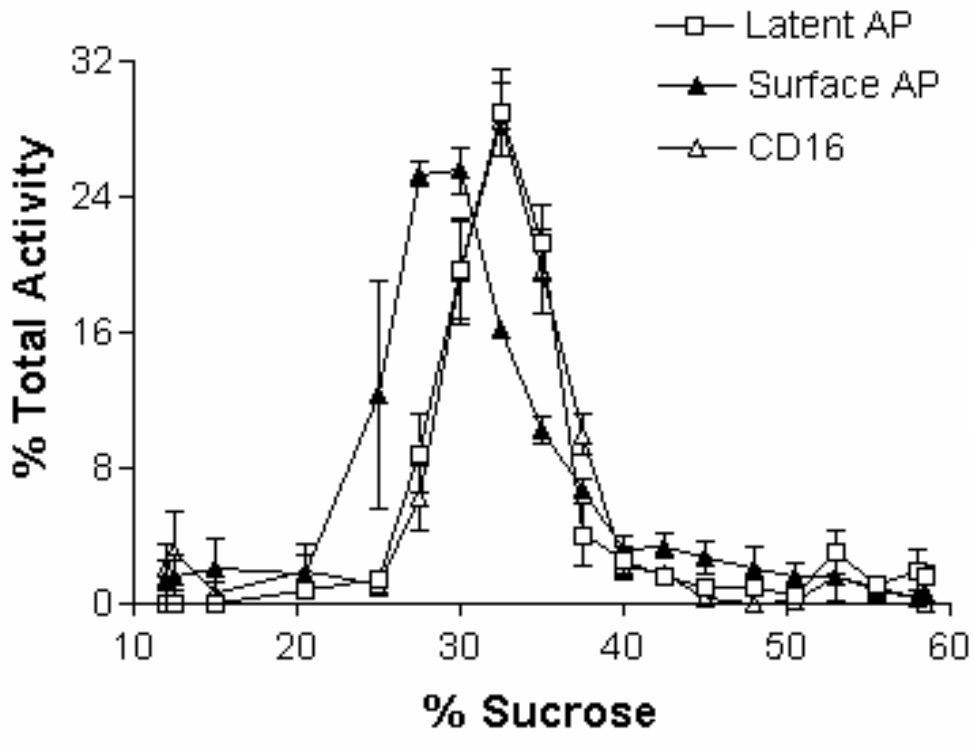

Latent AP
Surface AP
CD16
% Total Activity
% Sucrose
0
8
16
24
32
10
20
30
40
50
60

Figure 3. Subcellular distribution of alkaline phosphatase in human neutrophil plasma membranes and secretory vesicles. A) Subcellular distribution patterns of total cellular (□) and surface-associated (■) alkaline phosphatase activity from dextran-prepared neutrophil populations and total cellular alkaline phosphatase activity from gelatin gradients (■). Data are representative of comparable experiments from eight gradients, four dextran and four gelatin. B) Comparison of surface-associated (▲) and latent alkaline phosphatase activity (calculated; □) with subcellular distribution of CD16 (Δ) in gradient fractions obtained from density-fractionated dextran-prepared populations. Data are combined from either six gradients (alkaline phosphatase) or three gradients (CD16). Latent activity was calculated as the difference between surface-associated and total cellular alkaline phosphatase. Recovery of alkaline phosphatase and CD16 activities from gradient fractions was 87-95%. Error bars show standard error of the mean.

organization. Collectively, these studies raise questions concerning the extent of secretory organelle loss in gelatin-isolated neutrophil populations and further suggest possible nonspecific alterations in the surface membrane organization, shape, and/or structure of this population. We have addressed these questions, in part, by quantitatively demonstrating the absence of secretory vesicles in gelatin-isolated neutrophils (see above).

A prominent concern highlighted by one of the reports discussed above is the apparent capacity of neutrophil secretory vesicles to undergo a type exocytosis following cell stimulation that is similar to the compound exocytic behavior of eosinophils (McLaren et al., 1977) and mast cells (Guo et al., 1998). The resulting incomplete surface integration of this organelle subset would be expected to alter both the structural complexity and physical properties of plasma membranes (Scepek and Lindau, 1993) and, consequently, affect density sedimentation profile of plasma membrane marker activities (Mukherjee et al., 1994). We therefore examined the density sedimentation pattern of AP in gelatin-isolated populations relative to dextran-isolated controls. Measurement of this marker activity in either the absence of detergent (dextran

populations, Fig 3a) or in the absence and presence of detergent (gelatin-isolated populations, Figures 2b and 3a, respectively) invariably corresponded with each other as well as with other plasma membrane-specific markers, including Mg^{++} ATPase (compare Figures 1b and 3a), HLA and AMP-5' NT (compare Figures 1c and 3a). Accordingly, our data indicate the uniform cell surface integration of AP activity, and thus secretory organelle membranes, following their complete loss in gelatin-isolated populations.

To further verify the surface accessibility of AP activity, neutrophils were labeled with radioiodinated wheat germ agglutinin, [^{125}I]WGA, a lectin that binds with high affinity to certain cell surface glycoproteins, prior to cavitation. The subcellular distribution of [^{125}I]WGA consistently corresponded with cellular AP activity of gelatin-isolated (compare Figures 2c and 3a) as well as the detergent-free activity of dextran-isolated populations (Fig 2c), confirming the cell surface localization of this enzymatic activity in both populations. Notably, these data also demonstrate the utility of AP as a plasma membrane marker for subcellular mapping of plasma membrane enrichment in density gradient fractions prepared from gelatin-isolated neutrophil populations, irrespective of detergent usage.

In gradient fractions obtained from dextran-isolated neutrophil populations, peak distribution of AP activity shifted to approximately 3% greater density when assayed in the presence of detergent (Fig 3a). This finding is in agreement with previous work employing Percoll as density medium indicating secretory organelles to be slightly heavier in average density than plasma membrane-enriched vesicles (Borregaard et al., 1990). In contrast, gradient fractions from gelatin-isolated neutrophils *gave*

indistinguishable activity profiles in the presence and absence of detergent (Fig 3a). The density-shift in latent activity present in dextran populations was further examined by calculating the subcellular distribution of intact secretory vesicles. Our calculations confirm peak secretory organelle localization at 33% sucrose with substantial overlap at peak plasma membrane density of 30% sucrose (Fig 3b). This localization pattern was confirmed by examining the distribution of CD16, which, like AP, is a GPI-linked protein that is preferentially enriched in secretory organelles (Detmers et al., 1995). As shown in Figure 3b, the peak activity of CD16 coincides with the profile obtained for intact secretory vesicles, thus confirming the subcellular location of this organelle relative to peak plasma membrane activity.

As the study of plasma membrane biology is relevant to many fields of neutrophil research, a rapid and convenient method of isolating plasma membranes that is effective and widely accessible would facilitate research in many areas of investigation. The gelatin-isolation procedure thus offers a relatively rapid method for obtaining secretory vesicle-free neutrophil populations for use in density fractionation experiments to obtain purified plasma membrane-enriched vesicle populations. On the other hand, we surmise that the significant and differential impact of various neutrophil isolation methods on cellular complement of secretory vesicles demonstrated in this study could explain conflicting data obtained by different groups investigating various aspects of neutrophil physiology. Studies employing dextran-isolated neutrophil populations, for example, have shown that secretory vesicles house a significant fraction of cellular formyl peptide receptor (FPR) (Sengelov et al., 1994) and cytochrome b_{558} (Sengelov et al., 1992).

Secretory organelle loss could therefore contribute, directly and also indirectly by priming cells for further degranulation (Sengelov et al., 1993), to substantial variations in the number of cell surface-associated FPR reported in previous studies from gelatin-isolated (Sklar et al., 1984) versus dextran-isolated (Williams et al., 1977) neutrophil populations. Other studies examining the effector potential of intact secretory vesicles within 'resting' dextran-isolated neutrophils suggest that, upon stimulation with fMLF, initial activation of the NADPH oxidase occurs predominantly within this compartment and that cell-surface upregulation of this organelle confers on plasma membranes the capacity to generate superoxide (Kobayashi et al., 1998). These findings in combination with data presented in the current study suggest a physiologic basis for conflicting findings regarding the membrane specificity of NADPH oxidase assembly and activation found in neutrophils isolated by the gelatin method (Parkos et al., 1985) versus the dextran method (Borregaard et al., 1983). In addition, findings from the current study directly address the physiologic relevance of specialized subcellular compartments described previously in the context of FPR regulation (Jesaitis et al., 1986;Jesaitis et al., 1993;Jesaitis et al., 1989;Jesaitis et al., 1988), since the 'unstimulated' neutrophil populations employed in these latter studies were isolated by the gelatin method. Cell-surface upregulation of secretory vesicles has been intimately and inextricably linked with cellular priming (Morgan et al., 1997;Borregaard et al., 1994;Borregaard et al., 1987). Loss of this organelle in gelatin-isolated neutrophils therefore supports a cellular basis of priming in this population. Residual quantities of LPS in combination with incubation conditions required for gelatin-mediated red cell sedimentation may

additionally confer an LPS-primed cellular state (Dahinden et al., 1983;Dahinden and Fehr, 1983;Haslett et al., 1985). Therefore, study of plasma membranes bearing the full complement of secretory vesicle membrane-bound components should prove an important system for examining molecular mechanisms of neutrophil activation and effector-response coupling at the surface membrane level.

LITERATURE CITED

Balke N, Holtkamp U, Horl WH, and Tschesche H. 1995. Inhibition of degranulation of human polymorphonuclear leukocytes by complement factor D. FEBS Lett 371:300-302.

Bjerrum OW and Borregaard N. 1990. Mixed enzyme-linked immunosorbent assay (MELISA) for HLA class I antigen: a plasma membrane marker. Scand J Immunol 31:305-313.

Borregaard N. 1997. Development of neutrophil granule diversity. Ann N Y Acad Sci 832:62-68.

Borregaard N, Christensen L, Bejerrum OW, Birgens HS, and Clemmensen I. 1990. Identification of a highly mobilizable subset of human neutrophil intracellular vesicles that contains tetranectin and latent alkaline phosphatase. J Clin Invest 85:408-416.

Borregaard N and Cowland JB. 1997. Granules of the human neutrophilic polymorphonuclear leukocyte. Blood 89:3503-3521.

Borregaard N, Heiple JM, Simons ER, and Clark RA. 1983. Subcellular localization of the b-cytochrome component of the human neutrophil microbicidal oxidase: translocation during activation. J Cell Biol 97:52-61.

Borregaard N, Kjeldsen L, Sengelov H, Diamond MS, Springer TA, Anderson HC, Kishimoto TK, and Bainton DF. 1994. Changes in subcellular localization and surface expression of L-selectin, alkaline phosphatase, and Mac-1 in human neutrophils during stimulation with inflammatory mediators. J Leukoc Biol 56:80-87.

Borregaard N, Miller LJ, and Springer TA. 1987. Chemoattractant-regulated mobilization of a novel intracellular compartment in human neutrophils. Science 237:1204-1206.

Condliffe AM, Kitchen E, and Chilvers ER. 1998. Neutrophil priming: pathophysiological consequences and underlying mechanisms. Clin Sci (Lond) 94:461-471.

Dahinden C and Fehr J. 1983. Granulocyte activation by endotoxin. II. Role of granulocyte adherence, aggregation, and effect of cytochalasin B, and comparison with formylated chemotactic peptide-induced stimulation. J Immunol 130:863-868.

Dahinden C, Galanos C, and Fehr J. 1983. Granulocyte activation by endotoxin. I. Correlation between adherence and other granulocyte functions, and role of endotoxin structure on biologic activity. J Immunol 130:857-862.

DeChatelet LR and Cooper MR. 1970. A modified procedure for the determination of leukocyte alkaline phosphatase. Biochem Med 4:61-68.

DePierre JW and Karnovsky ML. 1973. Plasma membranes of mammalian cells: a review of methods for their characterization and isolation. J Cell Biol 56:275-303.

Detmers PA, Zhou D, Powell D, Lichenstein H, Kelley M, and Pironkova R. 1995. Endotoxin receptors (CD14) are found with CD16 (Fc gamma RIII) in an intracellular compartment of neutrophils that contains alkaline phosphatase. J Immunol 155:2085-2095.

Emmelot P and Bos CJ. 1966. Studies on plasma membranes. 3. Mg2+-ATPase,(Na+-K+-Mg2+)-ATPase and 5'-nucleotidase activity of plasma membranes isolated from rat liver. Biochim Biophys Acta 120:369-382.

Faurschou M and Borregaard N. 2003. Neutrophil granules and secretory vesicles in inflammation. Microbes Infect 5:1317-1327.

Guo Z, Turner C, and Castle D. 1998. Relocation of the t-SNARE SNAP-23 from lamellipodia-like cell surface projections regulates compound exocytosis in mast cells. Cell 94:537-548.

Guthrie LA, McPhail LC, Henson PM, and Johnston RB, Jr. 1984. Priming of neutrophils for enhanced release of oxygen metabolites by bacterial lipopolysaccharide. Evidence for increased activity of the superoxide-producing enzyme. J Exp Med 160:1656-1671.

Harlan J, DeChatelet LR, Iverson DB, and McCall CE. 1977. Magnesium-dependent adenosine triphosphatase as a marker enzyme for the plasma membrane of human polymorphonuclear leukocytes. Infect Immun 15:436-443.

Haslett C, Guthrie LA, Kopaniak MM, Johnston RB, Jr., and Henson PM. 1985. Modulation of multiple neutrophil functions by preparative methods or trace concentrations of bacterial lipopolysaccharide. Am J Pathol 119:101-110.

Henkel RD, VandeBerg JL, and Walsh RA. 1988. A microassay for ATPase. Anal Biochem 169:312-318.

Henson PM and Oades ZG. 1975. Stimulation of human neutrophils by soluble and insoluble immunoglobulin aggregates. Secretion of granule constituents and increased oxidation of glucose. J Clin Invest 56:1053-1061.

Jesaitis AJ, Bokoch GM, Tolley JO, and Allen RA. 1988. Lateral segregation of neutrophil chemotactic receptors into a. J Cell Biol 107:921-928.

Jesaitis AJ, Erickson RW, Klotz KN, Bommakanti RK, and Siemsen DW. 1993. Functional molecular complexes of human N-formyl chemoattractant receptors and actin. J Immunol 151:5653-5665.

Jesaitis AJ, Naemura JR, Painter RG, Sklar LA, and Cochrane CG. 1982. Intracellular localization of N-formyl chemotactic receptor and Mg2+ dependent ATPase in human granulocytes. Biochim Biophys Acta 719:556-568.

Jesaitis AJ, Naemura JR, Painter RG, Sklar LA, and Cochrane CG. 1983. The fate of an N-formylated chemotactic peptide in stimulated human granulocytes. Subcellular fractionation studies. J Biol Chem 258:1968-1977.

Jesaitis AJ, Tolley JO, and Allen RA. 1986. Receptor-cytoskeleton interactions and membrane traffic may regulate chemoattractant-induced superoxide production in human granulocytes. J Biol Chem 261:13662-13669.

Jesaitis AJ, Tolley JO, Bokoch GM, and Allen RA. 1989. Regulation of chemoattractant receptor interaction with transducing proteins by organizational control in the plasma membrane of human neutrophils. J Cell Biol 109:2783-2790.

Kobayashi T and Robinson JM. 1991. A novel intracellular compartment with unusual secretory properties in human neutrophils. J Cell Biol 113:743-756.

Kobayashi T, Robinson JM, and Seguchi H. 1998. Identification of intracellular sites of superoxide production in stimulated neutrophils. J Cell Sci 111 (Pt 1):81-91.

McLaren DJ, Mackenzie CD, and Ramalho-Pinto FJ. 1977. Ultrastructural observations on the in vitro interaction between rat eosinophils and some parasitic helminths (Schistosoma mansoni, Trichinella spiralis and Nippostrongylus brasiliensis). Clin Exp Immunol 30:105-118.

Morgan CP, Sengelov H, Whatmore J, Borregaard N, and Cockcroft S. 1997. ADP-ribosylation-factor-regulated phospholipase D activity localizes to secretory vesicles and mobilizes to the plasma membrane following N-formylmethionyl-leucyl-phenylalanine stimulation of human neutrophils. Biochem J 325 (Pt 3):581-585.

Mukherjee G, Quinn MT, Linner JG, and Jesaitis AJ. 1994. Remodeling of the plasma membrane after stimulation of neutrophils with f-Met-Leu-Phe and dihydrocytochalasin B: identification of membrane subdomains containing NADPH oxidase activity. J Leukoc Biol 55:685-694.

Parkos CA, Cochrane CG, Schmitt M, and Jesaitis AJ. 1985. Regulation of the oxidative response of human granulocytes to chemoattractants. No evidence for stimulated traffic of redox enzymes between endo and plasma membranes. J Biol Chem 260:6541-6547.

Philips MR, Abramson SB, Kolasinski SL, Haines KA, Weissmann G, and Rosenfeld MG. 1991. Low molecular weight GTP-binding proteins in human neutrophil granule membranes. J Biol Chem 266:1289-1298.

Quinn MT, Parkos CA, and Jesaitis AJ. 1989. The lateral organization of components of the membrane skeleton and superoxide generation in the plasma membrane of stimulated human neutrophils. Biochim Biophys Acta 987:83-94.

Scepek S and Lindau M. 1993. Focal exocytosis by eosinophils--compound exocytosis and cumulative fusion. EMBO J 12:1811-1817.

Sengelov H, Boulay F, Kjeldsen L, and Borregaard N. 1994. Subcellular localization and translocation of the receptor for N-formylmethionyl-leucyl-phenylalanine in human neutrophils. Biochem J 299 (Pt 2):473-479.

Sengelov H, Follin P, Kjeldsen L, Lollike K, Dahlgren C, and Borregaard N. 1995. Mobilization of granules and secretory vesicles during in vivo exudation of human neutrophils. J Immunol 154:4157-4165.

Sengelov H, Kjeldsen L, and Borregaard N. 1993. Control of exocytosis in early neutrophil activation. J Immunol 150:1535-1543.

Sengelov H, Nielsen MH, and Borregaard N. 1992. Separation of human neutrophil plasma membrane from intracellular vesicles containing alkaline phosphatase and NADPH oxidase activity by free flow electrophoresis. J Biol Chem 267:14912-14917.

Sklar LA, Finney DA, Oades ZG, Jesaitis AJ, Painter RG, and Cochrane CG. 1984. The dynamics of ligand-receptor interactions. Real-time analyses of association, dissociation, and internalization of an N-formyl peptide and its receptors on the human neutrophil. J Biol Chem 259:5661-5669.

Tschesche H, Kopp C, Horl WH, and Hempelmann U. 1994. Inhibition of degranulation of polymorphonuclear leukocytes by angiogenin and its tryptic fragment. J Biol Chem 269:30274-30280.

Ward RA, Nakamura M, and McLeish KR. 2000. Priming of the neutrophil respiratory burst involves p38 mitogen-activated protein kinase-dependent exocytosis of flavocytochrome b558-containing granules. J Biol Chem 275:36713-36719.

Williams LT, Snyderman R, Pike MC, and Lefkowitz RJ. 1977. Specific receptor sites for chemotactic peptides on human polymorphonuclear leukocytes. Proc Natl Acad Sci U S A 74:1204-1208.

Wilson ME, Jones DP, Munkenbeck P, and Morrison DC. 1982. Serum-dependent and -independent effects of bacterial lipopolysaccharides on human neutrophil oxidative capacity in vitro. J Reticuloendothel Soc 31:43-57.

CHAPTER 3

REORGANIZATION OF THE NEURTROPHIL CORTEX IS ASSOCIATED WITH FUNCTIONAL PRIMING WITHOUT ADHESION OR MORPHOLOGIC POLARIZATION

Abstract

We used human blood neutrophils, prepared by a gelatin-based method, as a novel model system for simulating the expected pathophysiologic conditions responsible for neutrophil priming *in vivo*. We used this methodology to isolate human neutrophil suspensions in an activated, pro-adherent cellular state that is highly transient *in vivo*. A pyrogen-free dextran-based method was used to acquire unprimed control neutrophils for comparison. Gelatin-prepared neutrophils were functionally primed for adherence and agonist-stimulated superoxide generation relative to unprimed control neutrophils. Cortical composition and structure of both neutrophil population was examined on subcellular fractions, Triton X-100-insoluble membrane skeleton preparations, and by immunofluorescence cytochemistry. Cortical proteins, F-actin, fodrin, and the fodrin-anchor, CD45 are largely cytoplasmic or intracellular in unprimed neutrophils but translocate to plasma membranes upon priming. Equilibrium sedimentation further revealed a lateral segregation of surface-associated cortical components (F-actin, fodrin, ezrin and associated anchors, CD43, CD45) relative to plasma membrane alkaline phosphatase. Our results suggest that cortical reorganization is coupled with the functional conversion of suspended blood-neutrophils and occurs independently of adhesive processes and morphologic polarization.

Abbreviations

ABTS, 2,2'-azino-bis [3-ethylbenziazoline-6-sulfonic acid]; AP, alkaline phosphatase; DPBS , Delbecco's phosphate-buffered saline; $DPBS^{4+}$, DPBS supplemented with 0.9 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1% bovine serum albumin and 0.1% dextrose; ERM, ezrin-radixin-moesin; fMLF, N-formyl-methionyl-leucyl-phenylalanine; EDTA, ethylene-diaminetetraceticacid-disodium-salt-dihydrate; GPI- glycophosphatidylinositol; HEPES, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; ME, 2-mercapto-ethanol; PMA, phorbol 12-myristate 13-acetate; PMSF, phenyl-methylsulphonyl-fluoride; PVDF, polyvinylidene fluoride membranes; SOD, superoxide dismutase; SV, secretory vesicle.

Introduction

Neutrophils are the first blood-phagocytes to arrive at extravascular sites of bacterial or fungal infection (Seely et al., 2003;Kobayashi et al., 2005). Once there, they prevent microbial colonization by the processes of phagocytosis, degranulation, and superoxide generation. The selective, ordered upregulation of multiple, distinct granule subsets before and after neutrophils arrive at extravascular sites of infection is a basic feature of the neutrophil-antimicrobial response (Sengelov et al., 1993a;Sengelov et al., 1995). Neutrophil granule populations, which include primary (azurophilic) and secondary (specific) granules, are initially derived from Golgi membranes during myelopoiesis (Borregaard et al., 1993a;Borregaard, 1996). By contrast, a third subset of vesicles in the neutrophil microbicidal arsenal, secretory vesicles (SV), are initially derived from the plasma membrane just before or shortly after mature neutrophils leave

the bone marrow (Borregaard et al., 1990;Faurschou and Borregaard, 2003). SV's are distinct from other subcellular endocytic fractions in that they are inherently stable within circulating, nonactivated blood-neutrophils and do not recycle with plasma membranes (Borregaard et al., 1992;Calafat et al., 1993;Borregaard et al., 1993b).

The ordered exocytosis of granule subpopulations appears to correspond with progressive stages of neutrophil activation (Baehner and Boxer, 1979;Gabig, 1980;Sengelov et al., 1993b;Borregaard et al., 1994). The first definable event in neutrophil activation is the transition of neutrophils from nonactivated to primed cellular states (Meldrum et al., 1997;Yamashiro et al., 2001). Priming is considered an intermediate state that markedly enhances the functional response of cells upon secondary stimulation (Kitchen et al., 1996;Condliffe et al., 1998). Neutrophil activation studies indicate that SV exocytosis precedes or coincides with neutrophil priming *in vitro*, thus suggesting that loss of this organelle may accompany the initial functional conversion of circulating blood-neutrophils upon agonist-activation, *in vivo* (Borregaard, 1996;Morgan et al., 1997). Once activated, circulating neutrophils must interact with and firmly adhere to activated endothelial cells. Firmly adherent neutrophils then leave the vasculature, transforming to a migratory stage, which enables their entry into infected tissues by the process of chemotaxis (Kvietys and Sandig, 2001). At the infected site, the exocytosis of primary and secondary granules initiates the microbicidal stage of neutrophil function. In particular, the delivery of granule components to developing phagosomes during neutrophil-ingestion of opsinized pathogen provides a basis for

microbial destruction by a plethora of oxygen-dependent and oxygen-independent mechanisms (Sawyer et al., 1989).

The microbicidal phase of neutrophil function has been investigated in many *in vitro* studies, which have elucidated specific changes in cellular biochemistry associated with agonist- stimulation and the priming process (McPhail et al., 1984;Niwa et al., 2004;Brown et al., 2004;Cadwallader et al., 2004;Guichard et al., 2005;Dang et al., 2006). Data from such studies indicate that the neutrophil-primed state may be rapidly acquired upon the exposure of resting cells to proinflammatory products, which can then markedly enhance cellular capacity for primary/secondary granule exocytosis, superoxide production and/or the generation of lipid mediators upon subsequent stimulation. However, *in vitro* studies are limited in that neutrophil priming behavior is examined in isolated systems of purified agonist and chemically defined buffer solutions, instead of the complex pathophysiologic microenvironment presented *in vivo*. Indeed, while *in vitro* studies imply the relative stability primed-neutrophils in suspension (Condliffe et al., 1998;MacKinnon et al., 2002;Liu et al., 2005), *in vivo* studies examining the functional conversion of circulating neutrophils in response to the infusion of proinflammatory products indicate the highly interactive and pro-adherent nature of this cellular state (Worthen et al., 1986;Haslett et al., 1987;Worthen et al., 1987;Young et al., 1990). Thus, upon agonist-exposure *in vivo*, circulating neutrophils rapidly adhere to vascular walls and subsequently leave the vasculature. Considered together, these data indicate that cellular priming prepares neutrophil for key stages of neutrophil function, including endothelial adherence and microbicidal function, and further suggest that priming *in vivo*

results in a pro-adherent phase that is rapidly manifested by a dynamic, highly transient population of agonist-activated, pre-adherent circulating neutrophils.

The loss of SV's is concomitant with neutrophil-priming *in vitro* and results in a marked increase in surface expression of β_2 integrins required for neutrophil-endothelial adhesion *in vivo* (Calafat et al., 1993;Sengelov et al., 1993b;Borregaard et al., 1994;Stefanidakis et al., 2004). SV's are additionally enriched in flavocytochrome b (Sengelov et al., 1992), chemoattractant receptors (Sengelov et al., 1994) and other signaling proteins. We have previously described two preparative methods yielding purified blood-neutrophils that either largely retain SV's or altogether lack SV's (Stie J and Jesaitis A.J, 2005). In particular, we found that neutrophil purification by a commonly used dextran-based method led to the preservation of SV's, while the use of a less common gelatin-based approach resulted in the selective loss of this organelle. Although the stimulus responsible for SV loss in gelatin-neutrophils is unknown, the isolation conditions imposed by this procedure simulate a pathophysiologic microenvironment compositionally similar to what might be expected within vascular regions adjacent to infected tissues. Specifically, gelatin is made from porcine skin and includes bacterial products. In the gelatin procedure, concentrated, suspended blood cells are incubated at 37ºC in the presence of gelatin. As such, this suspension is enriched in leukocytes, platelets, red cells, plasma proteins and proinflammatory mediators. The gelatin method may therefore be useful in acquiring blood-neutrophils that occupy the primed, pre-adherent state that is otherwise highly transient *in vivo*.

In this study, we use gelatin-neutrophils as an *in vitro* model system for the primed, pre-adherent state of *in vivo* primed neutrophils. We characterize the functional state and plasma membrane organization gelatin-neutrophils relative to nonactivated control cells isolated by the dextran method. We examined basal cellular adherence to plastic substrate and the respiratory burst in response to the proinflammatory chemotactic peptide, fMLF. We also determined the subcellular localization of transmembrane components of the membrane skeleton (CD45, CD43) as well as the peripherally bound cortical components (actin, fodrin, ezrin) in sucrose gradient-separated membrane fractions, detergent-insoluble membrane skeletons, and fixed intact and permeabilized neutrophils. We conclude that gelatin-prepared neutrophils are indeed primed and in contrast to dextran-prepared, unprimed neutrophils demonstrate an assembled cortical membrane skeleton that may structurally segregate the plasma membranes into lateral domains depleted in surface alkaline phosphatase but enriched in membrane skeletal proteins.

Materials and Methods

Materials

Chemicals were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise indicated. Several mouse anti-human monoclonal antibodies (Mabs) were used in these studies, including anti-actin C4 clone (ICN Biomedicals; Aurora, OH), anti-CD43 and anti-CD45 (Serotec Industries; Raleigh, NC), anti-ezrin (Neomarkers; Fremont, CA), anti-α_2 spectrin, and anti-β_2 spectrin (BD Transduction Laboratories;

Rockville, MD); anti-gp91^{phox} Mab 54.1 and the anti-p22^{phox} Mab 44.1 (recognizing epitope on the intracellular aspect of flavocytochrome b) are produced in this laboratory; the anti-gp91^{phox} Mab 7D5 (recognizing an extracellular epitope of the flavocytochrome b) was the kind gift of Dr. Michio Nakamura; bovine rhodopsin Mab K16 (Adamus et al., 1991) was used in these studies as an irrelevant control. Secondary HRP- and FITC-conjugated goat anti-mouse IgG antibodies were purchased from BioRad Laboratories (Hercules, CA) and Bethyl Laboratories, Inc. (Montgomery, TX), respectively. Other antibodies were obtained from the investigators producing them and are so referenced. FITC-phalloidin was provided by Dr. H. M. Miettinen.

Blood-Neutrophil Isolation

Neutrophils were isolated by either a dextran- or gelatin-based methodology, as previously described (Stie J and Jesaitis A.J, 2005). Briefly, isolation by the dextran-based procedure was performed using endotoxin-free reagents and materials following aseptic handling procedures. All solutions and buffers were made using WFI-grade distilled water (Hyclone, Logan UT). Initial red blood cell sedimentation was by 1:1 incubation of blood and saline with 3% dextran T-500 at room temperature for 30 min. The leukocyte-enriched supernatant was then centrifuged at 740 x g for 10 min, resuspended in 35 ml saline and underlaid with 10 ml Ficoll-Hypaque medium (< 0.01 ng/ml endotoxin) for gradient sedimentation 20 min at 415 x g. The resulting neutrophil-rich pellet was resuspended in ice-cold WFI-grade distilled water for 20 seconds to facilitate red blood cell lysis, immediately equilibrated to isotonicity by addition of an equivalent volume of 300 mM NaCl solution, and sedimented as above. Cells were

finally resuspended in 25 ml Dulbecco's phosphate buffered saline supplemented with 0.9 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1% bovine serum albumin and 0.1% dextrose ($DPBS^{4+}$), and treated with 3 mM di-isopropylfluorophosphate (DFP) for 15 min on ice. Prior to DFP addition, an aliquot of cells was removed for cell count determination by hemocytometer. Cells were then washed in the same buffer, resuspended in cavitation buffer (0.34 M sucrose, 10 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), pH 7.4, 1 mM ethylene-diaminetetraceticacid-disodium-salt-dihydrate (EDTA), 0.1 mM $MgCl_2$, 1mM ATP, protease inhibitor cocktail (P8340), and 100 μM phenyl-methylsulphonyl-fluoride (PMSF; Calbiochem; La Jolla, CA)), and N_2 -cavitated at 450 psi for 15 min at 4˚C. Post nuclear fractions were then isolated by centrifugation at 890 x g for 10 min and loaded onto linear sucrose gradients.

For the gelatin-based procedure, one unit of whole blood was initially centrifuged at 260 x g for 20 min at room temperature. The leukocyte- and erythrocyte-enriched pellet fraction was then resuspended in 500 ml per unit blood of a prewarmed (37˚C) saline solution supplemented with 2% gelatin, transferred into an autoclaved 500 ml polypropylene funnel, and further incubated 30 min in a 37˚C chamber to facilitate red cell sedimentation. The neutrophil-enriched supernatant was centrifuged at 360 x g for 10 min and pellets resuspended in 75 ml per unit blood ice-cold ammonium chloride lysis buffer (155 mM NH_4Cl, 9.4 mM $NaHCO_3$, pH 7.4, and 130 μM EDTA) and incubated for 5 min on ice with occasional mixing. Cells were then washed 1-2 times, counted, DFP-treated, disrupted by N_2 cavitation, and prepared for isopycnic sucrose density sedimentation exactly as described above.

Isopycnic Sucrose Gradient Sedimentation

Reagents, cell treatment/preparation methods, sucrose gradients and sedimentation conditions were as previously described (Stie J and Jesaitis A.J, 2005). Briefly, 20% and 55% sucrose solutions, each made wt/wt in 10 mM HEPES, pH 7.4, were transferred into a 50 ml capacity gradient maker (Hoefer, Inc; San Francisco, CA) in connection with a parastaltic pump. A total gradient volume of 25 ml was added into 30 ml thick-walled polycarbonate ultracentrifugation tubes, with 11 ml each of 20% and 55% sucrose solutions and an additional 3 ml cushion of 60% sucrose. Gradients were poured at 4˚C and maintained at this temperature for 16-24 hrs prior to use. Following addition of 5 ml post-nuclear fraction, prepared by 890 x g sedimentation for 10 min (4˚C), gradients were centrifuged 3.5 hrs at 83,000 x g in a SW28 swinging bucket rotor (Beckman; Palo Alto, CA). Gradients were then fractionated from the bottom using an Auto Densi-Flow IIC fraction collector (HBI Technologies; Phoenix, AZ) with fractions subsequently stored at either at 4˚C for immediate use or at -70˚C for later use. Fractions stored at -70˚C were thawed rapidly in the presence of protease inhibitor cocktail (P8340) and 100 μM PMSF prior to analysis in order to minimize proteolysis.

Functional Assays

In order to evaluate the adherence capacity of neutrophil populations, it was essential to employ a substrate that did not induce changes in the activation-state of the plated cells upon cellular contact. Because neutrophil adherence to plastic has provided a longstanding measure of cellular adherence capacity without stimulating adherence of resting cells (Fehr and Jacob, 1977;Dahinden et al., 1983;Dahinden and Fehr, 1983), we

employed this substrate for the current study in assays performed and quantified as described by Fehr and Jacobs (Fehr and Jacob, 1977).

Superoxide generation was determined by measuring SOD-inhibitable reduction of cytochrome c in a SpectraMax 250 microtiter plate reader (Molecular Devices; Sunnyvale, CA). Five microliters of neutrophil suspension (10^8 cells/ml) was added to microtiter plate wells containing 180 µl assay buffer (47 mM NaH_2PO_4, 18 mM K_2HPO_4, 1 mM EGTA, 2 mM NaN_3, 200 µM cytochrome c). The stimulus, phorbol myristate acetate (PMA), directly activates neutrophil-NADPH oxidase assembly and activation (independent of receptor activation) and was used as a positive control. After 15 min incubation at 37˚C, reactions were initiated by addition of stimulus alone (either 100 nM PMA or 100 nM fMLF) or stimulus with 5 µl bovine liver superoxide dismutase (SOD; 12,000 U/ml). In order to control for the possibility of adhesion-induced, spontaneous superoxide generation during analysis (Ginis and Tauber, 1990), control wells were included that consisted of cells in assay buffer without additional stimulus or SOD and evaluated in parallel with test samples. Adhesion-induced superoxide generation was not observed under the assay conditions employed. The rate of superoxide generation was continuously monitored as the absorbance change at wavelength 550 nm for 5 min after stimulus addition (T = 0). Because the rates of PMA-stimulated superoxide production were quantitatively indistinguishable within error limits in dextran- and gelatin-prepared neutrophils (see Table I), data obtained from both populations were used to calculate the appropriate curve shown in Figure 1B.

Biochemical Assays

Protein and calibrated refractive index measurements were performed as described previously (Stie J and Jesaitis A.J, 2005).

Alkaline phosphatase (AP) activity was assayed in the presence and absence of 0.1% Triton X-100 as previously described. Latent activity is defined as the relative amount of protein activity that is topologically sequestered within sealed vesicles such as intact secretory organelles or granules (i.e., intracellular or intravesiclular). Thus latent activity was calculated as the total cellular activity measured in the presence of detergent (or after freeze-thaw treatment) minus surface accessible activity measured in the absence of detergent (or without freeze-thaw treatment) and was also as described (Stie J and Jesaitis A.J, 2005).

ELISA Detection Assays

An ELISA procedure was used to assay CD45 and CD43 activities. Assays were performed according to the method described (Stie J and Jesaitis A.J, 2005) using the same controls. Latent activity as defined and calculated above was also measured for these activities. Due to the Triton X-100 detergent-sensitivity of the CD45- and CD43-specific Mabs used, detergent was not employed in these assays. Instead, cellular membranes (present within fractions collected from density gradient sedimentation) were disrupted by the freeze-thaw method of Philips et al. (Philips et al., 1991), in the presence of 100 μM PMSF and protease inhibitor cocktail (P8340), prior to the coating of microtiter plate wells. The efficacy of granule/vesicle lysis by this freeze-thaw method was evaluated by comparing the latent AP activity (defined as described above) obtained

in the presence/absence of detergent with that obtained using the freeze-thaw methodology. The AP activity in dextran-neutrophils is predominantly intracellular (or intravesicular), so this population was used for the analysis. Comparison of latent activities showed little difference in the efficacy of freeze-thaw lysis versus detergent lysis (97 $\pm$ 3 efficient for n = 3; data not shown). Interestingly, when the anti-CD45 employed in these assays was used to stain the fixed, saponin-treated neutrophil populations used for indirect immunofluorescence, this Mab did not exhibit the low signal to noise ratio observed with Triton X-100 solubilized gradient fractions in ELISAs (see below).

Indirect Immunofluorescence

Freshly isolated neutrophils were treated with 3 mM diisopropylfluorophosphate in DPBS, pH 7.4, for 15 minutes at 4°C. Cells were washed in the same buffer and fixed with 3% paraformaldehyde (in PBS, pH 7.4) for 15 minutes at 25°C, washed several times in the same buffer. Note that sedimentation was performed at the speeds/time indicated in the Flow Cytometry experiments (see below). Fixed cells were then resuspended with PBS, pH7.4, and 0.2% gelatin, with or without 0.01% saponin (referred to as blocking buffer) and incubated overnight at 4°C. Incubations were performed in 100 ul reaction volumes using 10^6 neutrophils. Primary labeling employed 50 nM FITC-phalloidin, 8 ug fodrin Mab (specific for the β_2 chain), 6 ug of control antibody (surface control Mab 7D5 or cytoplasmic control Mab 54.1) or 3 ug ezrin Mab in blocking buffer. Mab 7D5 labeling experiments were performed on intact (nonpermeabilized) neutrophils and used as a surface control stain. Irrelevant Mab control or controls without primary

antibody indicated no fluorescence and are not shown. All other primary-Mabs or FITC-phalloidin labeling experiments were conducted on permeabilized neutrophils. After primary labeling, reactions were quenched with 4.5 ml ice cold blocking buffer with or without detergent as indicated above followed by 2 additional low speed washings. After Mab labeling, the goat anti-mouse antibody, anti-IgG-FITC (diluted 1/400 in blocking buffer, $\pm$ detergent as indicated above) and was added in reaction volumes of 100 ul, and quenched and washed as above. All antibody incubations were for 30 minutes at 37°C. Stained cells were finally resuspended in 3.5 ml PBS then distributed in 100 ul aliquots within mini-funnels attached to glass slides fitted into a Cytospin 2 centrifuge. Cells were then spun onto slides (500 x g, 5 min). 50 ul of Fluoromount-G (SouthernBiotec; Birmingham, AL) was added to reduce background and preserve signal. Glass coverslips were then added and cells viewed microscopically using a Zeiss Axioskop 2 plus instrument equipped with a CD camera to allow video imaging via microcomputer and AxioVision Rel. 4.4 software.

Flow Cytometry Analysis

The proportion of cellular CD45 localized to the cell surface of gelatin-neutrophils was examined using two different approaches. In the initial approach, surface membrane CD45 expression was examined before and after degranulation. Degranulated neutrophils were prepared by suspending cells at $1x10^8$ per ml in Dulbecco's phosphate buffered saline supplemented with 0.9 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1% bovine serum albumin and 0.1% dextrose ($DPBS^{4+}$), 80 μg catalase, 10,000 activity units of SOD (Calbiochem; La Jolla, CA), and 2 μg/ml cytochalasin B in a 50 ml conical tube.

Cytochalasin B (which prevents F-actin polymerization) disassembles the cytoskeleton to allow for the complete surface upregulation of granule populations during fMLF stimulation. After a 7 min incubation at 37˚C, 1 μM fMLF was added and incubation continued for an additional 3 min. Cellular activation was terminated by addition of a 3-fold volume of ice-cold $DPBS^{4+}$ with 2 mM EDTA, 100 μM PMSF and protease inhibitor cocktail (P8340) followed by sedimentation at 740 x g for 10 min. Neutrophils were then washed 1-2 times in $DPBS^{4+}$ and pellets finally resuspended in $DPBS^{4+}$ with 3% goat serum at 10^7 cells per ml, and 100 μl aliquots distributed into 5 ml Falcon tubes (Fisher Scientific; Pittsburg, PA) for immunolabeling. Buffer was then added containing either no primary antibody (control), or 2 μg each of the anti-CD45 Mab, irrelevant Mab K16 (specific for bovine rhodopsin) or intracellular Mab 44.1 (specific for the intracellular aspect of the cytochrome b_{558} component $p22^{phox}$, used to verify the plasma membrane integrity of the populations tested),. Afterwards, cells were incubated 30 min on ice. After several washes, a 1:200 dilution of secondary FITC-conjugated goat anti-mouse IgG antibody was added followed by incubation as described above. After 1-2 washes, cells were resuspended in 500 μl PBS, pH 7.4, and examined by flow cytometry as previously described (13). As all negative controls were quantitatively similar, only data from Mab K16 are included in Figures 4A-B.

Alternatively, CD45 expression was examined on dextran-prepared populations, either additionally treated 30 min at 37˚C in 150 mM NaCl with or without 2% gelatin, or maintained on ice. Neutrophils maintained on ice were used as a negative control to obtain basal values of cell surface CD45 expression. Each of these neutrophil populations

were incubated with Mab and prepared for FACS analysis as described above. Degranulated populations of dextran-prepared neutrophils (containing fully upregulated CD45) were prepared by stimulating neutrophil-suspensions with fMLF in the presence of cytochalasin B as described above to allow maximal surface-CD45 expression. Degranulated neutrophils were assayed in parallel with the other populations defined above and used as a basis to quantify CD45 expression in the neutrophil populations assayed (see legend of Figure 4B).

SDS-PAGE and Immunoblot Analysis

SDS-PAGE was conducted as previously described using 6% and 10% polyacrylamide slab gels. Gradient fractions were denatured in SDS sample buffer containing 4% (v/v) 2-ME at 90˚C for 5 minutes. Electrophoretic mobility of gradient samples was calibrated using Cambrex Pre-stained Standards (Rockville, ME). Following SDS-PAGE, proteins were transferred onto Immobilon-P polyvinylidene fluoride membranes (PVDF; Fisher Scientific; Pittsburg, PA) and prepared for immunoblot analysis as described (Stie J and Jesaitis A.J, 2005). Mabs employed with dilutions in parentheses included actin (1:14,000); ezrin (1:3000); or the α_2 or β_2 chain of non-erythrocyte spectrin (also referred to as fodrin; 1:5000). Regarding fodrin-staining, Mabs specific for either the α_2 or β_2 chain yielded identical staining patterns, so only data from one Mab (anti-β_2 chain) is presented in this study. Immunoblots were developed using the Supersignal West Pico reagent kit (Pierce Biotechnology; Rockford, IL). Chemiluminescent signal was quantified from optical scans of exposed film using ONE-Dscan/1-D Gel Analysis software for windows (Scanalytics, Inc; Fairfax, VA). The

primary and secondary antibody concentrations used were selected to yield optical density readings in the linear range of detection for each antibody.

Detergent-Solubility Analysis

The Triton X-100 detergent solubility properties of the actin-based cytoskeleton of each neutrophil population were evaluated according to the method of Watts and Howard (Watts and Howard, 1994) using a TL 100 ultracentrifuge (Beckman; Palo Alto, CA). The three subpopulations of cytoskeletal actin isolated included (i) detergent-insoluble, low-speed pellet fraction; (ii) detergent-soluble high-speed pellet; and (iii) high-speed supernatant fractions. Following isolation, the actin, fodrin and ezrin content of each fraction was analyzed and quantified by SDS-PAGE/immunoblotting as described above.

Results

Gelatin-Prepared Neutrophils are Functionally Primed

Because of the well known response characteristics of gelatin-neutrophils (Henson and Oades, 1973;Henson and Oades, 1975;Sklar et al., 1981;Sklar et al., 1984;Hyslop et al., 1984;Sklar and Oades, 1985;Omann et al., 1987;Klotz and Jesaitis, 1994) and assumptions about the exposure of these cells to known priming agents (Vosbeck et al., 1990), it has become accepted that they are functionally primed. However, a side by side functional comparison of dextran-neutrophils and gelatin prepared cells has never been reported. To confirm that these two previously (see introduction) well characterized neutrophil populations represent functionally unprimed

and primed neutrophil populations respectively, we examined how well these two cell populations adhered to uncoated plastic (a measure of basal expression of adhesion capacity without specific ligand interaction) and how they responded to chemoattractants with a respiratory burst (a measure of receptor-mediated NADPH oxidase activation). Figure 1A shows that only 2 $\pm$ 1% (n = 4) of the dextran-neutrophils were able to adhere to uncoated plates, where as under identical conditions, gelatin-neutrophils demonstrated nearly 40-fold higher adherence capacity with 78 $\pm$ 4% (n = 4) adherence of the total cells plated (n = 4; Figure 1a).

We also observed significant differences in relative activity when the response of both populations were examined for the formyl peptide-induced respiratory burst, as measured by the SOD-inhibitable reduction of cytochrome c. Figure 1B shows that both cell types exhibited transient respiratory bursts lasting less that 5 min when stimulated with 100 nM fMLF. The rate of superoxide generation induced by 0.1 uM phorbol myristate acetate (PMA), the positive maximal stimulus control, was equivalent in both gelatin- and dextran-prepared populations, exhibiting no decline in rate during the period of measurement and indicating that the maximal respiratory burst potential of both cell types was the same. Thus PMA-stimulated superoxide generation is displayed in Figure 1B as the averaged activity from both populations. As shown in Table 1, however, examination of initial rates induced by stimulation with fMLF differed five-fold, with gelatin-neutrophils able to produce superoxide during the first min of stimulation as well the maximal positive PMA control (~3.0 nmol/10^6 cells in response to either fMLF (n = 4) or PMA (n = 4)). Dextran-neutrophils, on the other hand, exhibited a markedly weaker

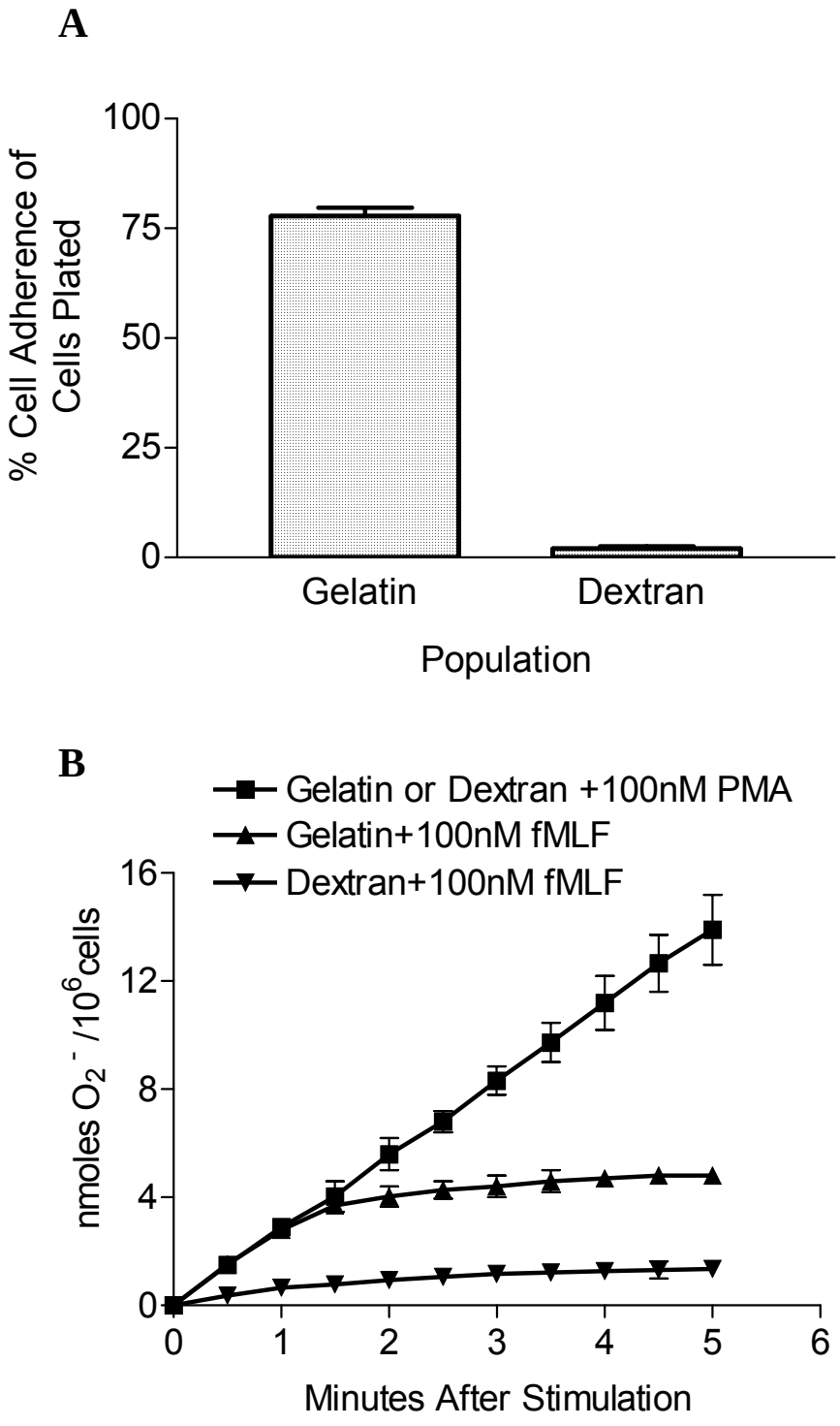


Figure 1. Comparison of adherence and fMLF-stimulated superoxide generation in dextran- versus gelatin-prepared neutrophils. A) Percent of gelatin- and dextran- prepared neutrophils adhering to plastic after settling out of suspension, measured and quantified

(Figure 1 –continued) as indicated in the Materials and Methods. B) The SOD-inhibitable cytochrome c reduction elicited over the first five minutes after cellular stimulation with 100 nM PMA (■) or 100 nM fMLF is indicated for gelatin- (▲) or dextran-prepared (▼) neutrophils. Because PMA-stimulated cellular activity was quantitatively indistinguishable between dextran- and gelatin-prepared neutrophils, data from both populations are represented in this curve. Each data set presented in A and B represents mean +/- standard error from 4 independent experiments.

response to fMLF of ~0.6 nmol/10^6 cells/min (n = 4; Table 1) or about one sixth the maximal rate induced by PMA. Without exception for both populations, we observed no increase in superoxide production in the absence of agonist addition (n = 8).

Table 1: Rates of superoxide generation in response to fMLF

Population	**PMA**	**fMLF**
Gelatin-Prepared	2.9 ± 0.1 (4)	2.8 ± 0.2 (4)
Dextran-Prepared	2.8 ± 0.1 (4)	0.6 ± 0.2 (4)

Data are expressed as nmol/10^6 cells/minute ± SEM (n) for the first minute following stimulation, after which time the fMLF-stimulated response of gelatin-prepared cells markedly declined (see Figure 1). Numbers in parenthesis indicate number of experiments. No oxidase activity was detected in adherent cells in the absence of activator.

Given the complex nature of the gelatin separatory milieu (see Introduction) it is clear that neutrophils prepared in this way are exposed to a variety of priming agents. Thus taken together, the above observed functional differences satisfy the consensus definition of the primed state in neutrophils: a cellular state demonstrating an amplified response to a stimulus caused by prior exposure to priming agent(s) (Swain et al., 2002). Therefore hereafter we will refer to gelatin-prepared neutrophils as "gelatin-primed".

Plasma Membrane Organization of Primed Versus Unprimed Human Neutrophils

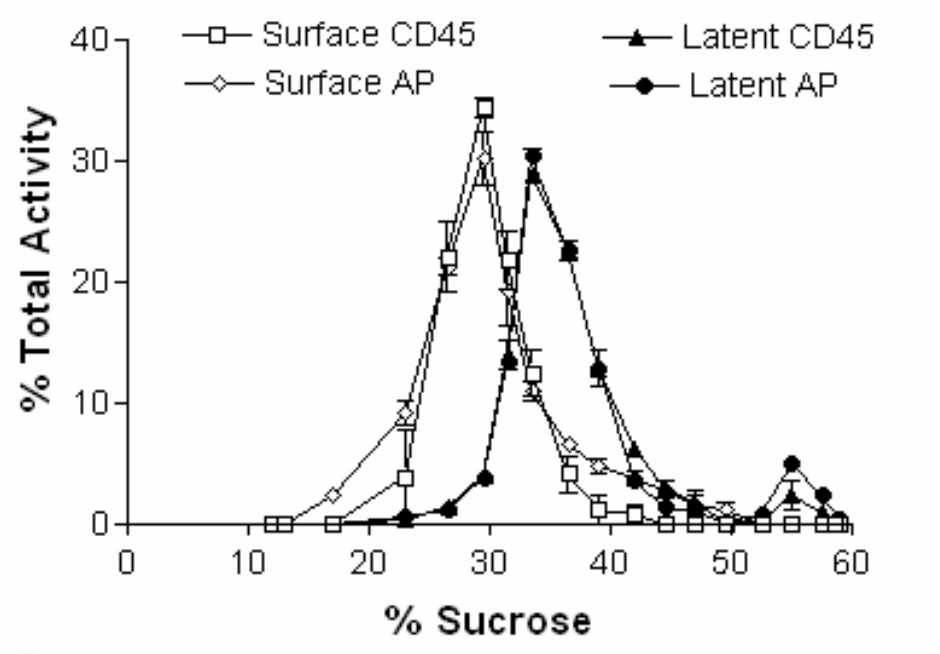
Another potential manifestation of the primed state is our recent observation that gelatin-prepared cells are devoid of secretory vesicles (SV), expressing no latent alkaline phosphatase activity and presumably other latent SV activities (see below). Incorporation of SV membrane contents into the surface plasma membrane results in changes in the composition of the surface membrane and, we hold, ultimately its lateral and transmembrane (including submembrane cortex) organization. Such changes can have profound effects on the function of fMLF-activated processes and have significant impact on fMLF stimulated superoxide production (Jesaitis et al., 1986;Jesaitis et al., 1988;Jesaitis et al., 1989;Quinn et al., 1989;Jesaitis et al., 1993;Jesaitis and Klotz, 1993).

To examine the state of organization of the plasma membrane and its membrane skeletal cortex, we compared plasma membrane subcellular fractions, isolated detergent-insoluble membrane skeletal fractions, and fixed and permeabilized intact neutrophils prepared by both gelatin (primed) and dextran (unprimed) methods for changes in composition and organization relative to specific membrane skeletal proteins. These key

proteins (actin, fodrin, CD45, ezrin and CD43) play an essential role in organizing the surface membranes of human leukocytes and the assembly and function of signaling complexes. Any significant difference in the organization of these proteins would suggest a contribution to regulation of the function of primed cells.

The subcellular localization of actin, fodrin, CD45, ezrin, CD43 and other activities (including alkaline phosphatase (AP) and CD16) were measured in gelatin-primed and dextran-unprimed neutrophil populations by isopycnic sucrose density gradient sedimentation of post nuclear supernatants from N_2 cavitated neutrophils prepared by both methods. The N_2 cavitation process is advantageous in this study because it selectively disrupts plasma membranes but leaves other organelles (e.g., secretory vesicles) intact. Previous reports examining the subcellular distribution of AP in density gradients have employed detergent-free AP activity as a plasma membrane-specific marker activity (Borregaard et al., 1990;Sengelov et al., 1992;Stie J and Jesaitis A.J, 2005). We have previously shown, in significant detail, that the distributions of detergent-free AP activity (referred to as surface AP) present in the primed and unprimed populations are *virtually identical*, but represent different percentages of the total activity (30% of dextran-unprimed and 100% of the gelatin-primed) in the two neutrophil populations (Stie J and Jesaitis A.J, 2005). These distributions, however, indicate the position of AP-enriched plasma membrane vesicles (i.e., surface AP) in such gradients and are plotted as a dashed average reference line in all figures (where appropriate) as a marker to indicate the density distribution of surface AP.

A



B

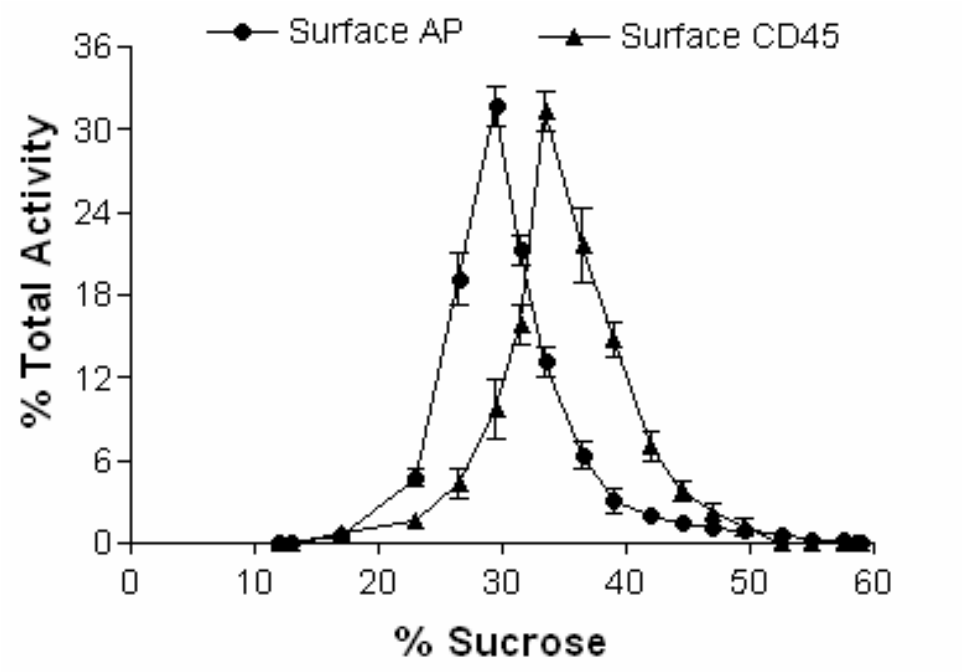


Figure 2. Distribution of CD45 and AP in sucrose density gradients made from gelatin-primed or dextran-unprimed neutrophils. In these studies, 5 x 10^8 neutrophils were

(Figure 2 –continued) disrupted by nitrogen cavitation and post nuclear fractions loaded onto linear sucrose gradients followed by equilibrium sedimentation. Data are shown as the percent total activity (in gradients) plotted as a function of percent sucrose concentration (wt/wt) of each fraction. A) CD45 and AP activities in gradient fractions prepared from dextran-unprimed neutrophils were measured before and after membrane disruption by either detergent-solubilization (AP) or freeze-thaw treatment (CD45). The activity measured before membrane disruption (Surface AP [Δ]) was subtracted from the activity measured after membrane disruption (Total activity, not shown) and the difference is plotted as the *latent* activity for each protein (Latent AP ● ; Latent CD45 ▲). B) CD45 and AP activities were measured in fractions prepared from gelatin-primed cells before and after membrane disruption and shown to be quantitatively *indistinguishable*. Thus latent activity was zero and only the Surface AP (●) and Surface CD45 (▲)in gradient fractions is plotted. Recovery of AP and CD45 activities from density gradients was greater than 90%. Error bars indicate standard error of the mean (SEM) from analysis of 3 gradients each from dextran-unprimed or gelatin-primed neutrophils (i.e. n=3).

CD45

CD45 is an integral membrane protein found in leukocytes that functions to anchor fodrin, the nonerythroid spectrin homolog, to the membrane in analogous fashion to BandIII/ankyrin complex anchoring spectrin in the erythrocyte membrane. Thus the distribution of CD45 should provide information about the distribution of fodrin anchorage sites in the plasma membrane of neutrophils. Figures 2A and 2B show the subcellular distributions of CD45 in density gradients prepared from dextran-unprimed and gelatin-primed neutrophils, respectively. These distributions were derived from indirect ELISA assays using an anti-CD45 monoclonal antibody on plates containing gradient fractions that were used directly or subjected to multiple freeze thaw cycles to break sealed vesicles as described in Material and Methods, due to detergent-sensitivity of the anti-CD45 Mab employed.

In density gradients prepared from dextran-unprimed neutrophils, we found that about 35 ± 5% (n=3) of the total cellular CD45 in gradient fractions could be measured prior to freeze-thaw with a peak in its distribution centered at 29% sucrose and paralleling the behavior of surface AP activity (Figure 2A). However, after freeze-thaw treatment, more activity was observed, at approximately 3 fold higher levels. When the total CD45 activity measured in the absence of freeze-thaw treatments was subtracted from the total respective activity measured after treatment and the difference plotted as *latent* CD45 activity, the distribution was indistinguishable from latent alkaline AP activity calculated in a similar way but using detergent treatment to disturb membranes. The peaks of both *latent* activities were clearly shifted by more that 5% sucrose to a higher density (34% sucrose), relative to surface AP activity. This distribution suggested that the latent CD45 was sequestered in denser SV as was the latent AP. Indeed a virtually identical *latent* activity profile with a peak distribution at ~34% sucrose was also found for another SV marker CD16, a glycophosphatidylinositol-linked Fc receptor (not shown).

By contrast, when density gradients prepared from gelatin-primed neutrophils were assayed directly or after membrane disruption, no *latent* CD45 or AP activities could be detected, suggesting both proteins are completely surface-accessible in this population. As expected for an exclusively surface protein, the subcellular distribution of total AP activity (assayed in the presence of detergent) aligned with surface AP (Figure 2B). Unexpectedly, however, the distribution profile of CD45 activity was shifted in peak

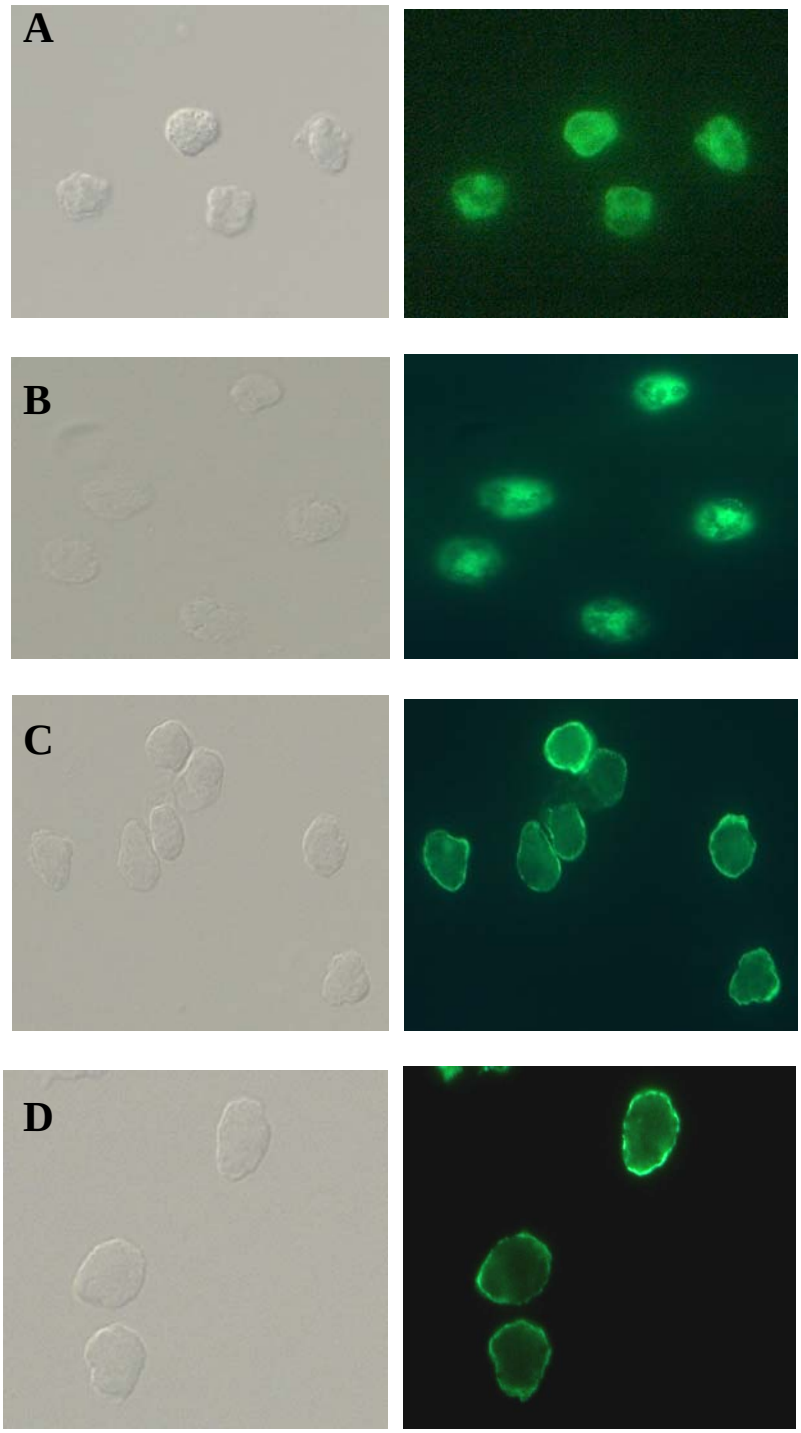


Figure 3. Analysis of cellular CD45 distribution in dextran-unprimed and gelatin-primed neutrophils by indirect immunofluorescence microscopy. Freshly isolated blood-neutrophils were immediately fixed and either permeabilized with saponin (A-C) or not

[Figure 3 –continued] (D) then labeled with the indicated Mabs as described in Materials and Methods. Each figure shows corresponding DIC (left) and fluorescence (right) images. A) Dextran-unprimed neutrophils labeled with anti-CD45 Mab show a largely intracellular cytoplasmic localization. B) Dextran-unprimed neutrophils labeled with Mab 54.1, that recognizes an intracellular epitope of neutrophil flavocytochromeb, shows a control cytoplasmic intracellular distribution. C) Gelatin-primed neutrophils labeled with anti-CD45 Mab, display a ringed fluorescence pattern typical of surface localization. D) Gelatin-primed neutrophils labeled with positive control Mab 7D5, that recognizes an extracellular epitope of neutrophil flavocytochrome b, shows a typical ring fluorescence pattern in intact (non-permeabilized) cells for comparison. Isotype matched irrelevant control antibody K16 (anti-bovine rhodopsin) or omitted primary antibody negative controls were both blank and are not shown.

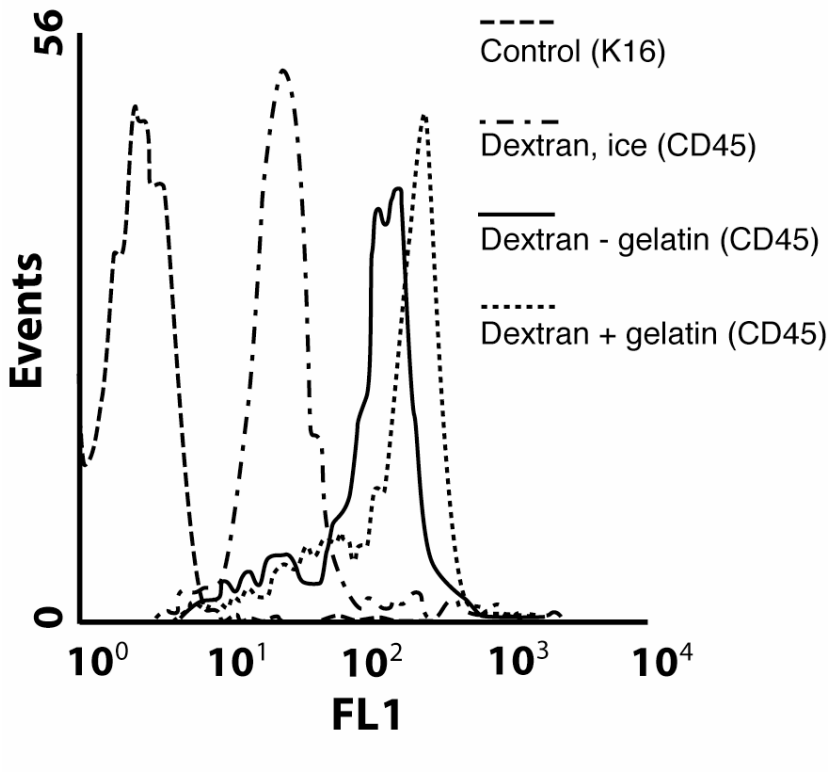
density relative to surface AP to ~34% sucrose. This shift in density was similar to that of *latent* CD45 activity (and SV) in dextran-unprimed neutrophils. Thus, despite the lack of detectable *latent* CD45 activity and the previously described absence of SV (Stie J and Jesaitis A.J, 2005) in gelatin-primed neutrophils, we cannot unequivocally conclude from this experiment that it is indeed at the cell surface.

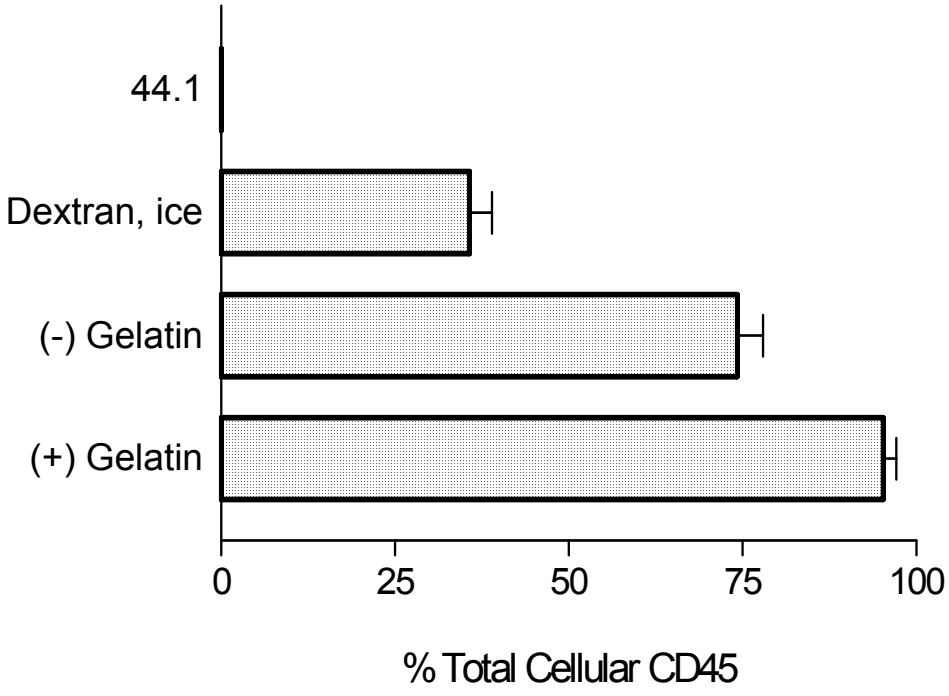
To independently show that CD45 is primarily localized on the cell surface of gelatin-primed neutrophils and is mostly sequestered to the cytoplasm in dextran-unprimed cells, we performed (n=2) indirect immunofluorescence analysis on fixed intact and permeabilized cells from both populations using an anti-CD45 Mab. Figure 3 shows that when permeablized cells of both types are prepared only gelatin-primed neutrophils (Figure 3C) display a classic ring fluorescence indicative of a surface localization. This distribution of stain is similar to that observed when the 7D5 antibody, which recognizes an extracellular epitope of flavocytochrome b (Burritt et al., 2001), was used to label the surface of *unpermeabilized* neutrophils (Figure 3D). In contrast, in dextran-unprimed neutrophils (Figure 3A), the anti-CD45 Mab displays a fluorescence pattern that is typically seen in cytoplasmic distributions such as that shown for a cytoplasmic epitope

of the anti-flavocytochrome b recognized by Mab 54.1 (Figure 3B). Such a distribution typically displays stain exclusion zones consistent with cytoplasmic nuclear lobes (Figures 3A-B). Controls using irrelevant K16 Mab as the primary antibody were devoid of fluorescence and are not shown.

To show that gelatin exposure could induce redistribution of CD45, a flow cytometry analysis was performed on dextran-unprimed cells with and without subsequent exposure to gelatin. In these experiments, dextran-unprimed neutrophils, which have minimal expression levels of CD45 on surface membranes, were either maintained on ice or incubated 30 min at 37˚C in saline with or without 2% gelatin. Cell surface CD45 expression was then examined by flow cytometry analysis. As indicated in Figure 4A, 95 ± 3% and 75 ± 5% of cellular CD45 was detected on the cell surface of neutrophil populations incubated in the presence and absence of 2% gelatin, respectively. Iced control populations, however, had a relatively low CD45 expression of 34 ± 5% (n = 3; Figure 4A). Surface up-regulation of CD45 in these experiments was calculated based on values obtained for activated/fully degranulated neutrophils (see below). These data suggest that neutrophil isolation under the conditions imposed by the gelatin procedure used in this study is sufficient to elicit a nearly maximal surface expression of cellular CD45. On the other hand, the partial (~75%) up regulation of CD45 observed after 37˚C incubation in saline without 2% gelatin is likely related to the temperature-dependent, spontaneous up regulation of secretory vesicles shown to occur upon exposure of unprimed neutrophils to elevated temperatures (Borregaard et al., 1987).

A


56
Events
0
Control (K16)
Dextran, ice (CD45)
Dextran - gelatin (CD45)
Dextran + gelatin (CD45)
10^0
10^1
10^2
10^3
10^4
FL1


44.1
Dextran, ice
(-) Gelatin
(+) Gelatin
0
25
50
75
100
% Total Cellular CD45

B

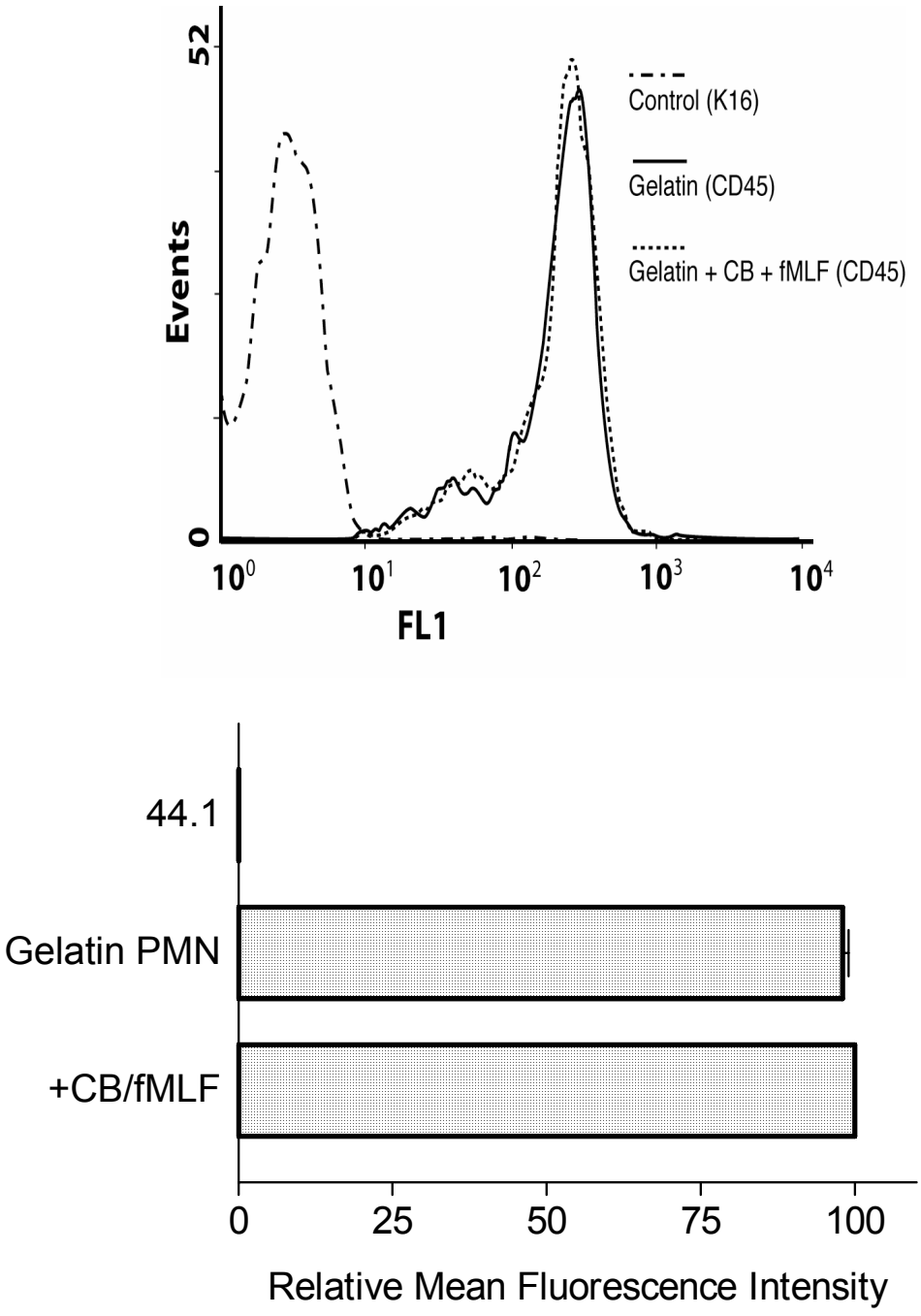


Figure 4. Analysis of stimulus-induced CD45 redistribution in gelatin-primed neutrophils by flow cytometry. A) Flow cytometry histogram (top) and bar graph (bottom) showing CD45 expression on dextran-prepared neutrophils either maintained on ice (Dextran, ice) or incubated at 37˚C in isotonic NaCl solution in the presence (Dextran + gelatin) or

(Figure 4 –continued) absence (Dextran - gelatin) of 2% gelatin. B) Flow cytometry histogram (top) and bar graph (bottom) showing CD45 expression on the surface of gelatin-primed neutrophils that were maintained on ice (Gelatin) or fully degranulated by stimulation with 1 μM fMLF in the presence of 2 μg/ml cytochalasin B (Gelatin + CB + fMLF). The bar graphs below the flow cytometry histograms in A and B show the mean fluorescence intensity of cell-surface CD45, calculated as percent of maximum intensity obtained from fully degranulated populations (+ CB/fMLF) and arbitrarily set at 100%. The average ± SEM of the data shown in each bar graph is averaged over three independent experiments. Mab K16 is specific for rhodopsin and included in experiments as irrelevant control (Control, K16). Staining with either irrelevant antibody (followed by secondary antibody) or secondary alone yielded identical curves only the former is shown.

Results from indirect immunofluorescence (Figure 3C) and flow cytometry (Figure 4A) studies suggested CD45 was predominantly intracellular in dextran-unprimed neutrophils but predominantly surface-associated in gelatin-primed neutrophils. To confirm that expression of CD45 on surface membranes of gelatin-primed neutrophils is maximal, this population was subjected to additional treatments and further examined by flow cytometry analysis. First, a positive control of activated/fully degranulated neutrophils (displaying maximal surface upregulation of CD45) was prepared by stimulating gelatin-primed neutrophils with fMLF in the presence of cytochalasin B. Previous studies have shown that such fMLF-stimulation in the presence of cytochalasin B results in the complete loss of neutrophil granules (Mukherjee et al., 1994). The mean fluorescence intensity of surface-associated CD45 was then compared between fully degranulated gelatin-primed neutrophils and untreated gelatin-primed neutrophils (Figure 4B). As indicated in Figure 4B, both gelatin-primed and gelatin-primed fully degranulated populations exhibited equivalent maximal mean fluorescence intensity for cell surface CD45 expression, relative to the dextran-unprimed control population,

providing further evidence that cellular CD45 is entirely plasma membrane associated in gelatin-primed neutrophils.

Fodrin

CD45 provides structural binding sites for fodrin, the $\alpha_2 \beta_2$ homolog of erythroid spectrin, in a functionally significant interaction in leukocytes (Bourguignon et al., 1985;Lokeshwar and Bourguignon, 1992). The redistribution of CD45 from a primarily cytoplasmic vesicular organization in dextran unprimed neutrophils to a cell-surface distribution in gelatin-primed neutrophils could suggest that an analogous redistribution pattern occurs for fodrin, in such neutrophil populations. Therefore, we further compared the subcellular distribution of fodrin in dextran-unprimed and gelatin-primed neutrophils.

As shown in Figure 5A, cellular fodrin of dextran-unprimed neutrophils was predominantly (~93%) found at or near the top of the sucrose gradients at 10-18% sucrose density, indicating cytosolic origin. By contrast, the distribution of fodrin in gradient fractions from gelatin-primed neutrophils was bimodal, with a minor peak (containing 30% of fodrin activity) at 18% sucrose and a major peak (containing 70% fodrin activity) at 33-36% sucrose. This latter peak activity at 33-36% sucrose in gelatin-primed neutrophils cosediments most closely with the CD45-enriched plasma membrane regions of these density gradients and is significantly shifted from the surface AP activity shown as a dashed reference line. The relative amount of fodrin cosedimenting within gradient regions of plasma membrane enrichment was calculated as $7 \pm 4\%$ and $70 \pm 6\%$ for dextran-unprimed and gelatin-primed neutrophils, respectively (Table 2).

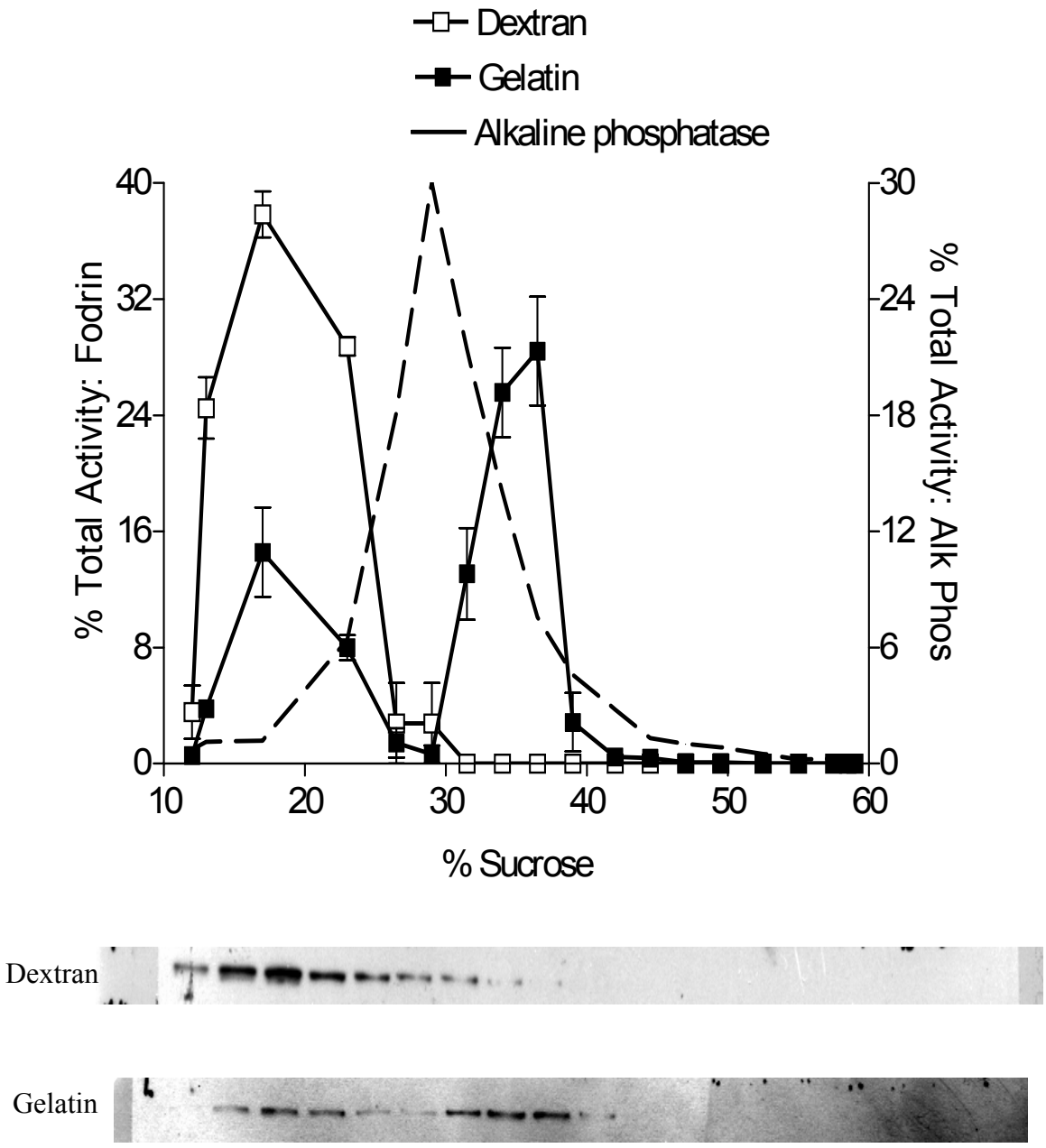


Figure 5. Comparison of fodrin subcellular localization in dextran-unprimed and gelatin-primed neutrophils. Neutrophils from each population were prepared and density-fractionated as described in the legend of Figure 2 and, more fully, in Material and Methods. Percent of the total activity in all gradient fractions is plotted as a function of sucrose percentage (wt/wt) in gradient fractions. Fodrin content in fractions from dextran-unprimed (□)and gelatin-primed (■) neutrophil populations, determined by quantitative

(Figure 5 –continued) immunoblotting with anti-fodrin β-chain Mab, is plotted. Below the figure are two films showing representative immunoblots of fodrin from dextran-unprimed (upper) and gelatin-primed (lower) neutrophil fractions. Surface AP (-----) is shown to identify the distribution of AP-enriched plasma membrane. Error bars indicate standard error of mean from analysis of three gradients from each cell population (i.e. n=3). Recovery of fodrin from density gradients exceeded 96% of the activity present in the original low speed supernatant prior to fractionation.

Table 2: Percent of total gradient content of actin and fodrin in plasma membrane-enriched fractions of sucrose density gradients from dextran-unprimed and gelatin-primed neutrophils

Component	**Population**	**% PM Localization**
Fodrin	Dextran	7 ± 4%
	Gelatin	70 ± 6%
Actin	Dextran	20 ± 3%
	Gelatin	35 ± 4%

Neutrophils isolated from donor blood by dextran- or gelatin-based techniques were cavitated and then fractionated by isopycnic sucrose density sedimentation as described in the Materials and Methods Section. Fodrin and actin localization within gradient regions of plasma membrane (PM)-enrichment, indicated by the averaged distribution of alkaline phosphatase in figures 2A (fodrin) and 3A (actin), is indicated. Results shown are from six independent experiments.

To confirm that the relative differences in fodrin distribution in dextran-unprimed and gelatin primed neutrophils represented redistribution in the cortical cytoskeleton,

detergent-insoluble membrane skeletal fractions from each neutrophil population were isolated and examined. Neutrophil membrane skeletons have characteristic detergent-insolubility properties that allow the isolation of this structure from other cytoskeletal pools (Watts and Howard, 1992;Watts and Howard, 1994). In this method the detergent-

Table 3: Analysis of cytoskeletal composition of *in vitro*-isolated membrane skeletons from dextran-unprimed and gelatin-primed neutrophils

Component	**Population**	**Actin-Pool** [£]		
		LSP	**HSP**	**HSS**
Fodrin	Dextran	15 ± 2%	28 ± 3%	57 ± 4%
	Gelatin	89 ± 4%	11 ± 3%	48 ± 4%
Actin	Dextran	19 ± 3%	33 ± 2%	48 ± 4%
	Gelatin	40 ± 3%	26 ± 2%	34 ± 3%
Ezrin	Dextran	100%	0	0
	Gelatin	100%	0	0

Neutrophils isolated from donor blood by dextran- or gelatin-based techniques treated as described in the Materials and Methods Section. Proteins were fractionated by SDS-PAGE and immunoblotted for fodrin, actin and ezrin using chemiluminescence. Chemiluminescent signal was quantified from optical scans of exposed film using ONE-Dscan/1-D Gel Analysis software for windows. [£]LSP, low-speed pellet (membrane skeleton); HSP, high-speed pellet (non-cortical, cytoplasmic actin-based polymeric networks) ; HSS, high-speed supernatant (monomeric actin). Results shown are from a total of four independent experiments.

insoluble fraction is collected by low speed sedimentation, and the remaining cytoskeletal pools are pelleted from the high-speed centrifugation of low speed supernatants. According to this approach, only ~15% of cellular fodrin isolated within the detergent-insoluble membrane skeleton of dextran-unprimed neutrophils (n = 2; Table 3). The low fodrin content of membrane skeletons prepared from dextran-unprimed neutrophils is thus consistent with density sedimentation data indicating sedimentation of ~93% cellular fodrin at sucrose densities substantially lighter than the plasma membrane compartment (n = 3; Table 2). By contrast, fodrin distribution in gelatin-primed neutrophils was largely insoluble, with 89 ± 4% cellular fodrin found in the cortical cytoskeleton (n = 2; Table 3). This data is therefore consistent with the sedimentation of ~70% cellular fodrin within plasma membrane-enriched regions in sucrose density gradients (n = 3; Table 2).

To independently confirm the cellular organization of fodrin in dextran-unprimed and gelatin-primed neutrophils, immunocytochemistry was also performed on these populations. Representative micrographs are shown in Figure 6 from at least two independent experiments per population. Accordingly, anti-fodrin Mab labeling of dextran-unprimed neutrophils (Figure 6A) corresponded with the cytoplasmic control pattern (Figure 6B) using Mab 54.1, both showing the nuclear stain exclusion zones as remarked on above for CD45 in dextran-prepared neutrophils. This distribution is in agreement with results obtained from both density sedimentation analysis (Figure 5) and membrane skeletal analysis (Table 3). In contrast, the immunofluorescence staining pattern detected in gelatin-primed neutrophils differed significantly, in that the fodrin staining produced a ring-like fluorescence around the cell periphery (Figure 6C), which

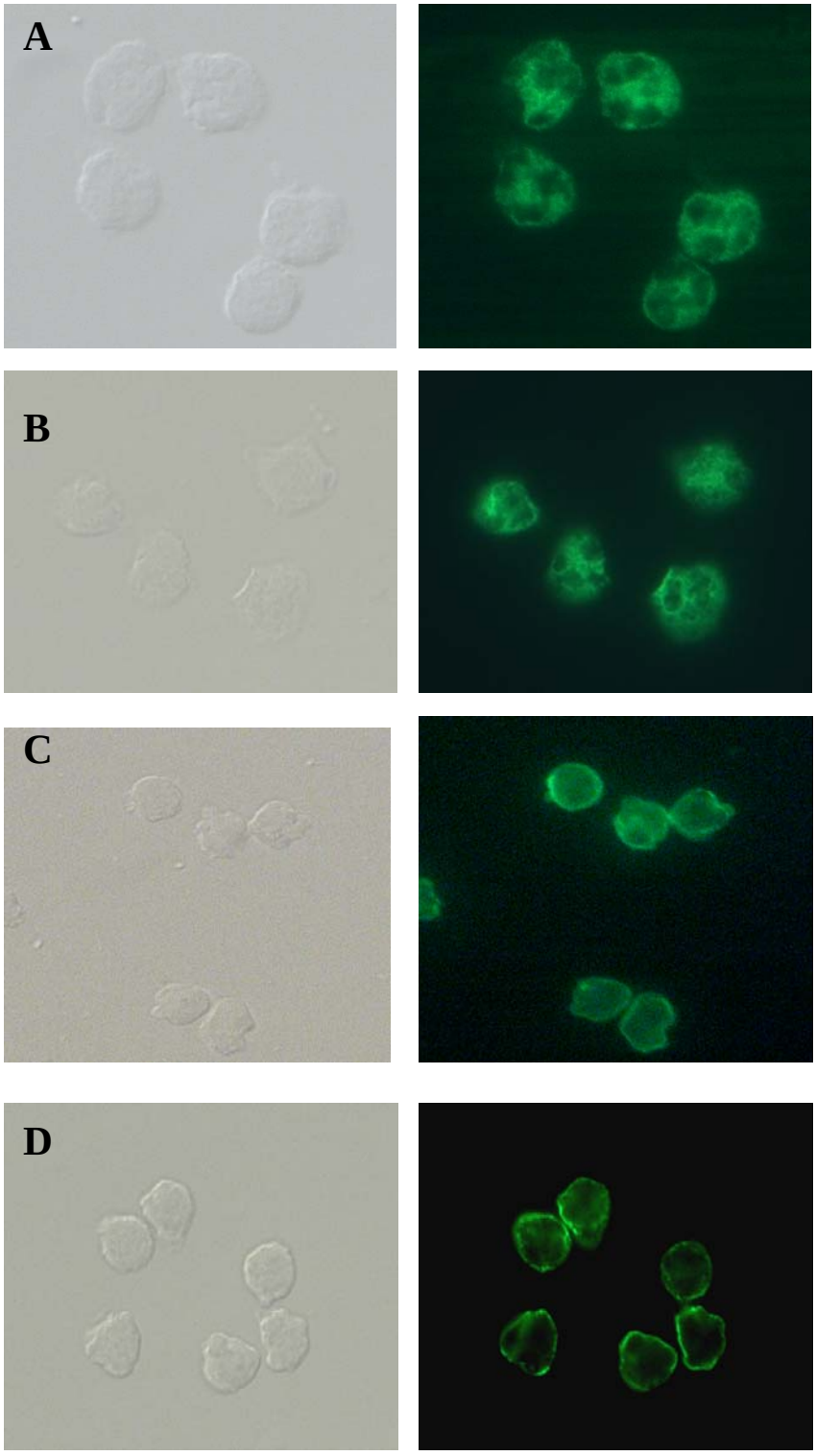


Figure 6. Analysis of cellular fodrin distribution in dextran-unprimed and gelatin-primed neutrophils by indirect immunofluorescence microscopy. Freshly isolated blood-

(Figure 6 –continued) neutrophils were immediately fixed and either permeabilized with saponin (A-C) or not (D) then stained with the indicated Mabs as described in Materials and Methods. Each figure shows corresponding DIC (left) and fluorescence (right) images. A) Dextran-unprimed neutrophils labeled with anti-fodrin β-chain Mab suggests a largely cytoplasmic localization. B) Dextran-unprimed neutrophils labeled with Mab 54.1, that recognizes an intracellular epitope of neutrophil flavocytochromeb, shows a control cytoplasmic intracellular distribution. C) Gelatin-primed neutrophils labeled with anti- fodrin β-chain Mab, display a ringed fluorescence pattern typical of surface localization. D) Gelatin-primed neutrophils labeled with positive control Mab 7D5, that recognizes an extracellular epitope of neutrophil flavocytochrome b, shows a typical ring fluorescence pattern in intact (non-permeabilized) cells for comparison. Isotype matched irrelevant control antibody K16 (anti-bovine rhodopsin) or omitted primary antibody negative controls were both blank and are not shown.

corresponded to that of the surface control stain (Figure 6D), and is consistent with fodrin incorporation into the plasma membrane-associated membrane skeleton. Taken together, the staining pattern of fodrin in gelatin-primed neutrophils coupled with the observed shift in CD45 (Figure 2B) and fodrin (Figure 5) distributions relative to surface AP in sucrose density gradients provides evidence suggesting the selective assembly of compositionally distinct plasma membrane microdomains in gelatin-primed neutrophils. Specifically, these data suggest that plasma membranes from primed, nonpolarized neutrophils can be regionally segregated into compartments that are either enriched or depleted in surface AP activity.

Actin-Based Cortical Cytoskeleton

Cortical actin associates with different families of integral membrane proteins in leukocytes, including the sialoglycoprotein CD43 (Yonemura et al., 1993;Serrador et al., 1998;Yonemura et al., 1998), through ezrin/radixin/moesin (ERM) linkages. As such, CD43 and ezrin activities can be considered cortical components. Like CD45 and fodrin, these components form a functional complex in leukocytes (analogous to the glycophorin

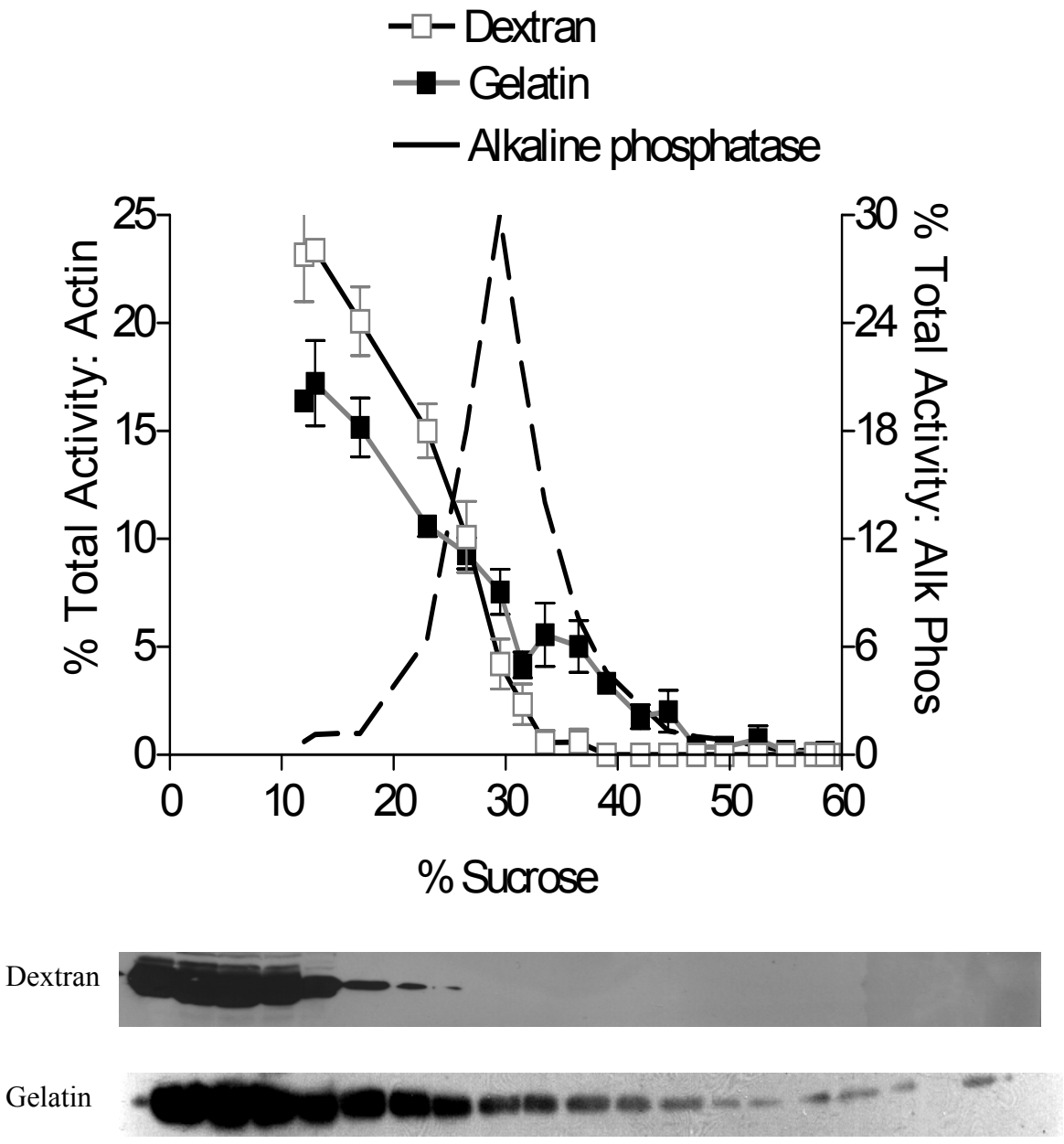


Figure 7. Comparison of subcellular actin localization in dextran-unprimed and gelatin-primed neutrophils. Neutrophils from each population were prepared and density-fractionated as described in the legend of Figure 2 and more fully in Material and Methods. Percent of the total activity in all gradient fractions is plotted as a function of sucrose percentage (wt/wt) in gradient fractions. Actin content in fractions from dextran-unprimed (□) and gelatin-primed (■) neutrophil populations, determined by quantitative

(Figure 7 –continued) immunoblotting with anti-actin Mab, is plotted. Below the figure are two films showing representative anti-actin immunoblots from dextran-unprimed (upper) and gelatin-primed (lower) neutrophil fractions . Surface AP (-----) is shown to identify the distribution of AP-enriched plasma membrane fractions. Error bars indicate standard error of mean from analysis of 3 gradients from each cell population (i.e. n=3). Recovery of actin from density gradients exceeded 96% of the activity present in the original low speed supernatant prior to fractionation.

C-protein 4.1-F-actin complex in erythrocytes) and may codistribute within the cortical structure of unprimed and primed neutrophils. We therefore used actin, CD43 and ezrin as marker activities to further examine cortical reorganization in the two populations of neutrophils.

The subcellular distribution of actin in sucrose density gradients prepared from gelatin-primed or dextran-unprimed neutrophils showed significant differences. The major peak activities as to be expected for monomeric and, possibly, macromolecular assemblies of actin were observed in the cytosol-enriched fractions, between 12-18% sucrose and declining (~25% per fraction to ~ 5% per fraction) with increasing density (Figure 7). However, the amount of actin localizing within the plasma membrane-enriched zone of the gradients of each population (as indicated by the traced distribution of surface AP) differed by approximately 2-fold and was 20 ± 3% and 35 ± 4% of total cellular actin for dextran-unprimed and gelatin-primed neutrophils, respectively (Table 2). Notably, in gelatin-primed neutrophils, the minor peak of actin sedimenting at ~33-36% sucrose (Figure 7) cosediments with the peak activities of fodrin (Figure 5) and CD45 (Figure 2B).

The cortical content of actin within gelatin-primed and dextran-unprimed neutrophils was further examined by determining the percentage of cellular actin present

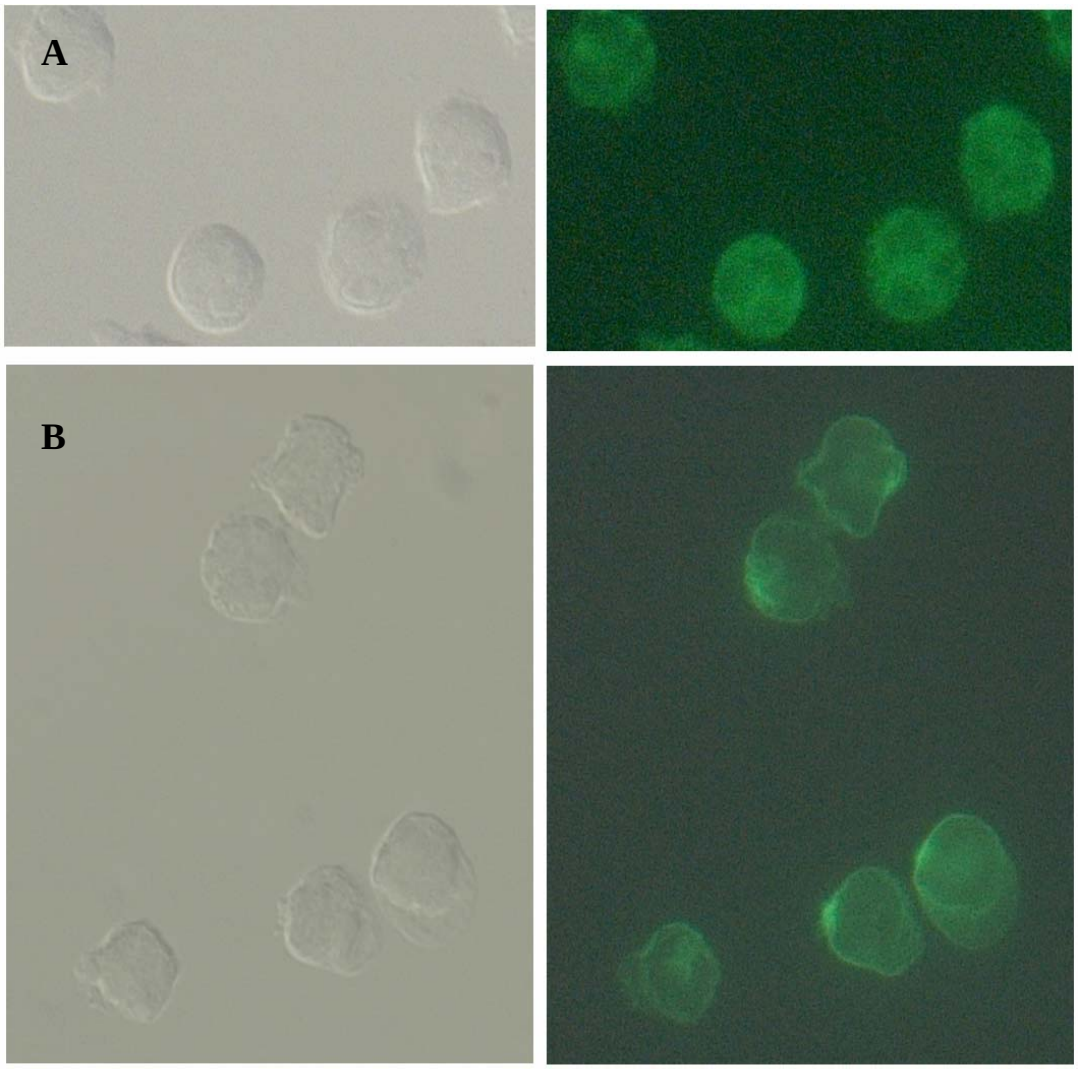


Figure 8. Analysis of cellular F-actin distribution in dextran-unprimed and gelatin-primed neutrophils by FITC-phalloidin fluorescence microscopy. Freshly isolated blood-neutrophils were immediately fixed and permeabilized with saponin then stained with FITC-phalloidin or control antibodies as described in Materials and Methods. Each figure shows corresponding DIC (left) and fluorescence (right) images. A) Dextran-unprimed neutrophils labeled with FITC phalloidin show a largely intracellular diffuse cytoplasmic localization. B) Gelatin primed- neutrophils labeled with FITC-phalloidin show a rimmed fluorescence pattern indicative of surface cortical localization. Intracellular and surface positive control staining patterns (carried out with Mabs 54.1 and 7D5 are not shown) but matched those shown in Figures 3 and 6. Irrelevant antibody and primary antibody omission controls were blank (not shown).

within *in vitro* isolated membrane skeletons analyzed as described above for fodrin. We found that the amount of cortical F-actin present in membrane skeletal fractions of dextran-unprimed neutrophils (19 ± 3%; n = 2; Table 3) was consistent with the actin levels found within the plasma membrane compartment of density gradients for this population (20 ± 3%; Table 2). Close to 2-fold increases were detected in cortical F-actin present in membrane skeletons from gelatin-primed neutrophils (40 ± 3%; n = 2; Table 3) and similar to the percentage of total cellular actin detected in the plasma membrane compartment of density gradients prepared from gelatin-primed neutrophils (35 ± 4%; Table 2).

Indirect immunofluorescence microscopy and FITC-phalloidin staining was used to further examine the cellular organization of filamentous actin (F-actin) within gelatin-primed and dextran-unprimed neutrophils. The labeling pattern of F-actin observed in dextran-unprimed neutrophils (Figure 8A) appeared to be restricted to the cell cytoplasm. This observation is consistent with the relatively low content of actin detected in the plasma membrane region of dextran-unprimed neutrophils in sucrose density gradients (Figure 7) and within *in vitro* isolated membrane skeletons (Table 3). However, the cellular organization of F-actin in gelatin-primed neutrophils differed dramatically. A partial ringed-fluorescence pattern was observed in gelatin-primed neutrophils and suggestive of a cortical distribution for F-actin in this population (Figure 8B). This staining pattern is consistent with those obtained for CD45 (Figure 3) and fodrin (Figure 6). It is also consistent with data from the subcellular fractionation studies of actin (Figure 7), as well as data from the analysis of membrane skeletal actin (Table 2),

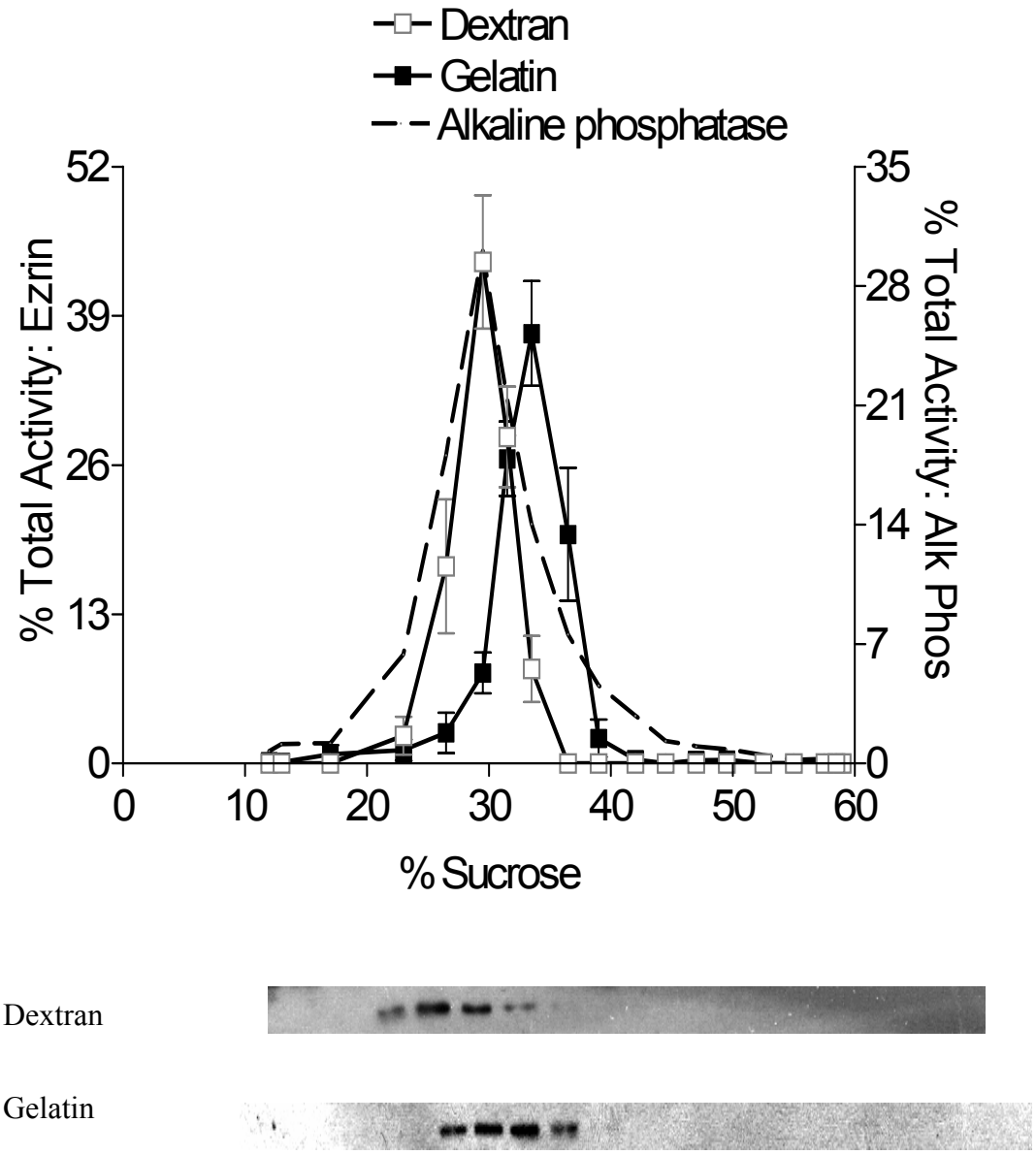


Figure 9. Comparison of ezrin subcellular localization in dextran-unprimed and gelatin-primed neutrophils. Neutrophils from each population were prepared and density-fractionated as described in the legend of Figure 2 and more fully in Material and Methods. Percent of the total activity in all gradient fractions is plotted as a function of sucrose percentage (wt/wt) in gradient fractions. Ezrin content in fractions from dextran-

(Figure 9 –continued) unprimed (□) and gelatin-primed (■) neutrophil populations, determined by quantitative immunoblotting with anti-ezrin Mab, is plotted. Below the figure are two films showing representative immunoblot of anti- ezrin immunoblots from dextran-unprimed (upper) and gelatin-primed (lower) neutrophil fractions . Surface AP (-----) is shown to identify the distribution of AP-enriched plasma membrane fractions. Error bars indicate standard error of mean from analysis of 3 gradients from each cell population (i.e. n=3). Recovery of ezrin from density gradients exceeded 96% of the activity present in the original low speed supernatant prior to fractionation.

suggesting that cortical actin comprised close to half the cellular actin in this neutrophil population. Considered together, these data further support the redistribution and segregation of cell-surface transmembrane and cortical proteins in gelatin-primed neutrophils.

When the subcellular distribution of ezrin was examined in sucrose density gradients prepared from dextran-unprimed neutrophils, we found that the peak activity of this protein coincided with that of surface AP in dextran-unprimed neutrophils at ~29% sucrose (Figure 9). By contrast, this activity was clearly shifted into a region of higher density at ~33% sucrose in gelatin-primed neutrophils (Figure 9). Importantly, the ezrin profile of gelatin-primed neutrophils coincided in relative peak density with other cytoskeletal activities of CD45 (Figure 3), fodrin (Figure 5) and actin (Figure 7). Virtually no ezrin activity was detected in the cytosol-enriched regions of density gradients prepared from either gelatin-primed or dextran-unprimed neutrophils, thus indicating that the cellular pool of ezrin is primarily plasma membrane-associated in both neutrophil populations. In agreement with these data, when *in vitro*-isolated membrane skeletons from each neutrophil population were prepared and analyzed for ezrin content, cellular ezrin was exclusively detected within the detergent-insoluble membrane skeletal fraction (Table 3). The cellular organization of ezrin was also examined by indirect

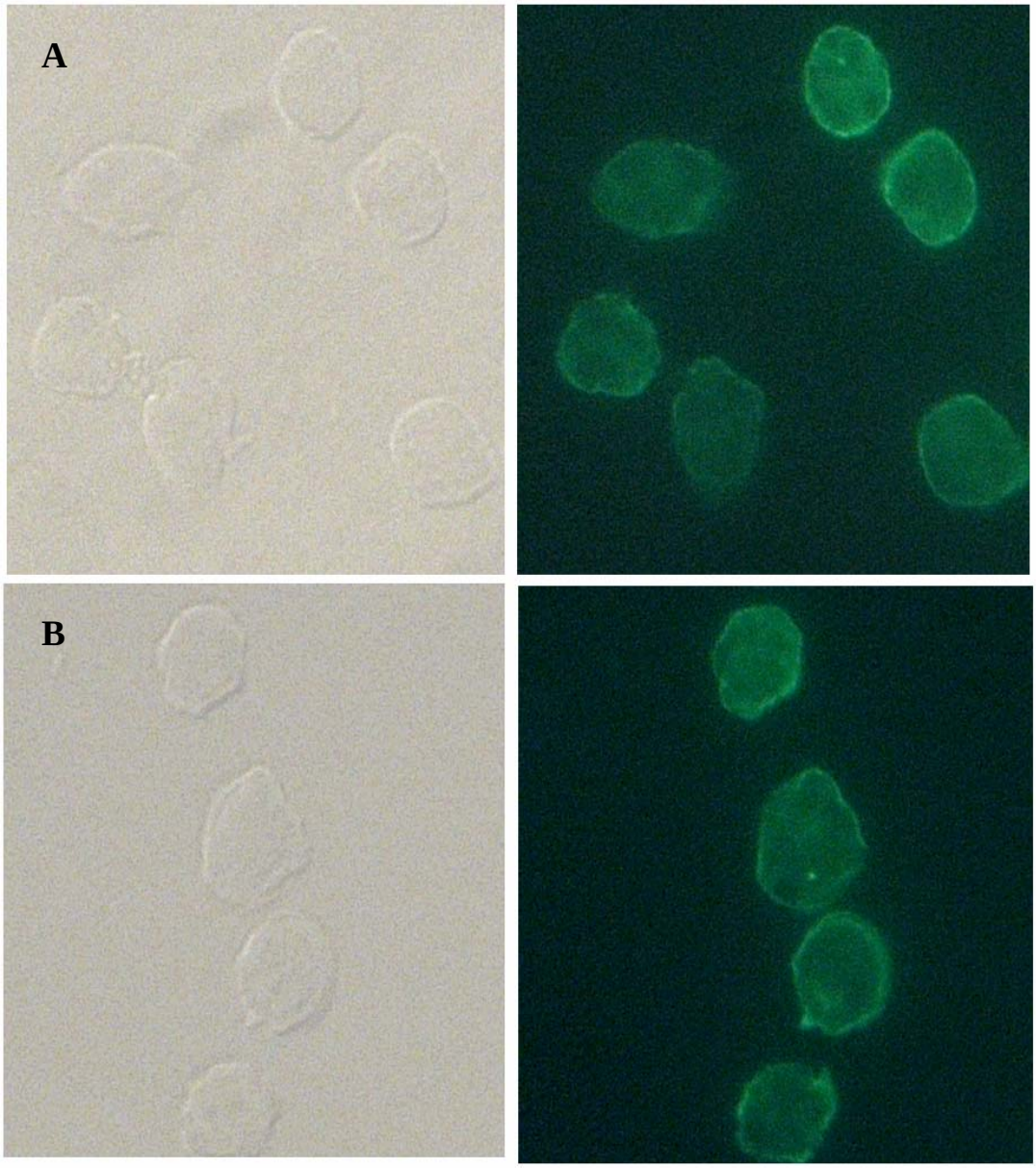


Figure 10. Analysis of cellular ezrin distribution in dextran-unprimed and gelatin-primed neutrophils by indirect immunofluorescence microscopy. Freshly isolated blood-neutrophils were immediately fixed and permeabilized with saponin and then labeled with anti-ezrin antibody as described in Materials and Methods. Each figure shows corresponding DIC (left) and fluorescence (right) images. A) Dextran-unprimed neutrophils labeled with anti-ezrin antibody display a ringed fluorescence pattern suggestive of a largely surface cortical localization. B) Gelatin-primed neutrophils

(Figure 10 –continued) labeled with anti-ezrin antibody display a rimmed fluorescence pattern indicative of a surface cortical localization. Intracellular and surface positive control staining patterns (carried out with Mabs 54.1 and 7D5 are not shown) but matched those shown in Figures 3 and 6. Irrelevant antibody and primary antibody omission controls were blank (not shown).

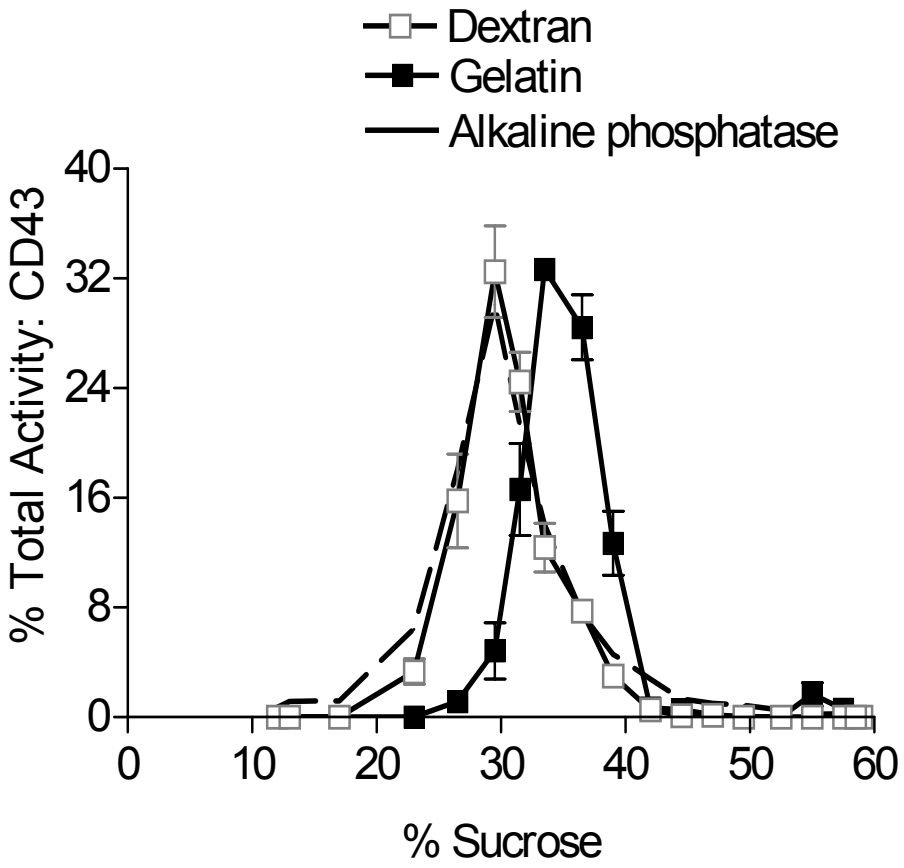


Figure 11. Comparison of CD43 subcellular localization in dextran-unprimed and gelatin-primed neutrophils. Neutrophils from each population were prepared and density-fractionated as described in the legend of Figure 2 and more fully in Material and Methods. Percent of the total activity in all gradient fractions is plotted as a function of sucrose percentage (wt/wt) in gradient fractions. CD43 content in fractions from dextran-unprimed (□) and gelatin-primed (■) neutrophil populations, determined by ELISA with anti-CD43 Mab, is plotted. Surface AP (-----) is shown to identify the distribution of AP-enriched plasma membrane fractions. Error bars indicate standard error of mean from analysis of 3 gradients from each cell population (i.e. n=3). Recovery of CD43 from density gradients exceeded 90% of the activity present in the original low speed supernatant prior to fractionation.

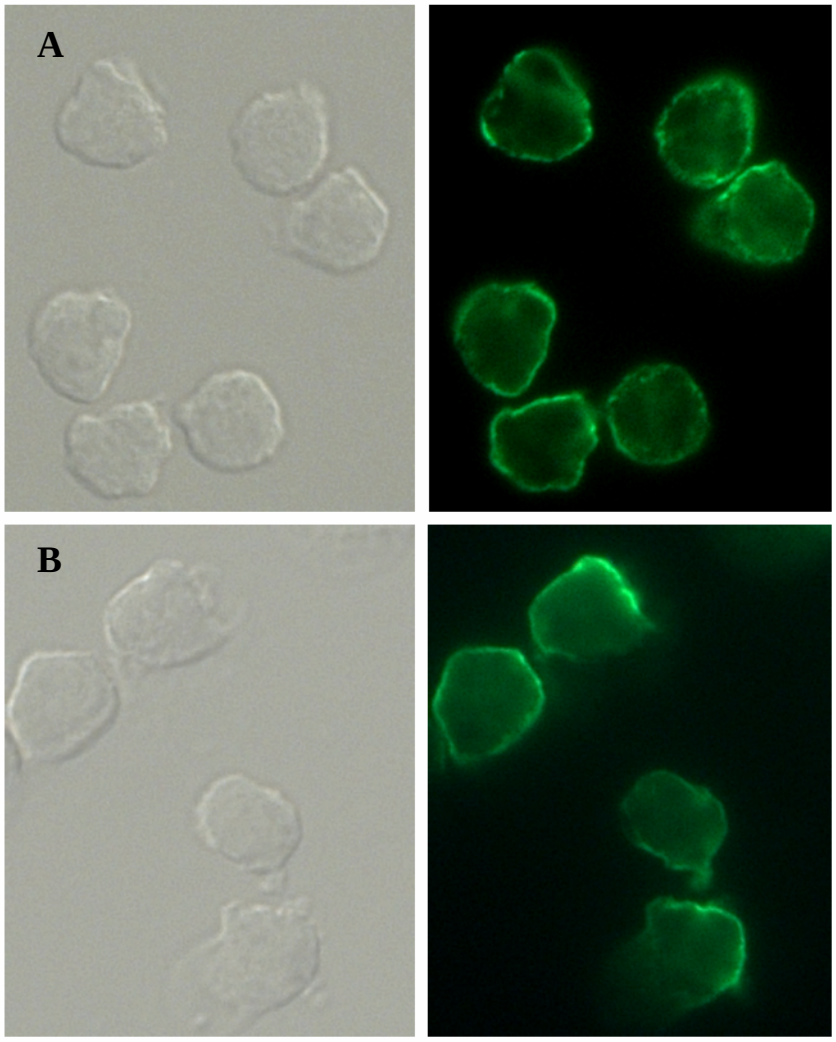


Figure 12. Analysis of cellular CD43 distribution in dextran-unprimed and gelatin-primed neutrophils by indirect immunofluorescence microscopy. Freshly isolated blood-neutrophils were immediately fixed and permeabilized with saponin and then labeled with anti-CD43 antibody as described in Materials and Methods. Each figure shows corresponding DIC (left) and fluorescence (right) images. A) Dextran-unprimed neutrophils labeled with anti-CD43 antibody display a ringed fluorescence pattern suggestive of a largely surface localization. B) Gelatin-primed neutrophils labeled with anti-CD43antibody display a rimmed fluorescence pattern indicative of a surface localization. Intracellular and surface positive control staining patterns (carried out with Mabs 54.1 and 7D5 are not shown) but matched those shown in Figures 3 and 6. Irrelevant antibody and primary antibody omission controls were blank (not shown).

immunofluorescence microscopy shown in Figure 10. The specific staining patterns of ezrin in both dextran-unprimed and gelatin-primed neutrophils revealed a tightly formed ringed fluorescence around the cell periphery, suggestive of its integration within the membrane skeleton (Figure 10A-B).

The subcellular distributions of ezrin (Figure 9) in dextran unprimed and gelatin-primed neutrophils also closely corresponded to that of CD43 (Figure 11), an ezrin-binding transmembrane protein (Yonemura et al., 1998). The cellular distribution of CD43 was also analyzed by indirect immunofluorescence microscopy and found to be exclusively on the surface of both dextran-unprimed and gelatin-primed neutrophils (Figures 12A-B). Overall, these data, in association with the shifted sedimentation patterns of CD43 and ezrin into surface AP-poor (gelatin-primed neutrophils) or surface AP-enriched (dextran-unprimed neutrophils) regions of sucrose density gradients provide additional evidence for the large-scale structural compartmentalization of surface membrane in gelatin-primed neutrophils.

Discussion

In this study, we have shown that gelatin-primed neutrophils are both pro-adherent and functionally primed for superoxide generation relative to dextran-unprimed neutrophils. Interestingly, as a result of gelatin-preparation, blood-neutrophils do not undergo a morphological polarization, in terms of pseudopod development, but instead acquire a uniformly ruffled, round, unpolarized morphology (Tolley et al., 1987). This contrasts with the microvilli that uniformly decorate the surface of nonadherent,

functionally inert blood neutrophils (Shao et al., 1998;Park et al., 2002). Thus, we suspected that the functional differences observed between dextran-unprimed and gelatin-primed blood-neutrophils might be associated with respective organizational differences in their plasma membrane structure.

Our attempts to examine this structure using equilibrium sedimentation analysis, cytoskeletal fractionation procedures, and quantitative and morphological examination of cells by immunoflourescence methods produced several unexpected findings. First, we found that the functional transition of blood-neutrophils from an unprimed state to the pro-adherent, functionally primed state of gelatin-prepared neutrophils was associated with large-scale changes in the subcellular distribution of cortical F-actin (Figures 7-8), actin binding protein ezrin (Figures 9-10), fodrin (Figures 5-6), and their respective transmembrane anchoring proteins, CD43 (Figures 11-12) and CD45 (Figures 2-3). Our approach to examining submembrane architecture was based on mapping the subcellular distribution of components contributing to the two major anchoring complexes that exist hematopoietic cell plasma membranes, including the CD43-ezrin-F-actin (Yonemura et al., 1993;Yonemura et al., 1998) complex and CD45-fodrin complex (Bourguignon et al., 1985). CD43 is the major transmembrane sialoglycoprotein species in leukocytes and is thus analogous to erythrocyte glycophorin C. Both proteins anchor to cortical F-actin through protein 4.l-like linkages (band 4.1 in erythrocytes, ezrin in leukocytes). Similarly, leukocyte CD45 is functionally and structurally associated with fodrin in leukocytes and, as such, is analogous to the band 3/ankyrin complex that anchors erythrocyte spectrin to the membrane. The observed cosedimentation of F-actin and fodrin with their respective

anchoring complexes in dextran-unprimed and gelatin-primed neutrophils is thus suggestive of the physiologic nature of the cortical reorganization patterns observed in this study.

Many previous neutrophil studies have examined the properties of cortical F-actin redistribution during agonist-activated mechanical responses occurring in association with phagocytosis of opsinized antigen (Valerius et al., 1981;Ryder et al., 1984;Zhelev and Hochmuth, 1995), substrate adhesion(Sullivan and Mandell, 1983;Bengtsson et al., 1993;Yeung et al., 2005), pseudopod development and/or directed motility (Cassimeris et al., 1990;Cano et al., 1991;Cano et al., 1992). The latter motility studies have largely focused on the redistribution properties of cortical F-actin, actin binding proteins and/or various transmembrane protein anchors during the course of cellular polarization leading to pseudopod development. The current study differs from this body of work in several important respects. First, gelatin-primed neutrophils lack polarized morphological structures associated with directed motility (e.g., pseudopods). Furthermore, because neutrophils are maintained in suspension throughout the gelatin procedure, they lack the extensive adhesion plaques typical of adherent populations. In effect, the gelatin-primed neutrophils may be a model system to examine a novel ‘snap-shot’ of the physical state of circulating neutrophils during their initial functional conversion *in vivo*. As such, our study affords new and significant insight into changes that the membrane and cytoskeleton undergo to achieve the primed state in the absence of polarization-inducing stimuli and the ensuing morphologic asymmetries that accompany pseudopod development.

In order to insure the physiologic nature of results described in this study, we examined the organization of cortical F-actin/fodrin skeletons using sucrose density sedimentation in the absence of detergent solubilization. In this way, the subcellular organization of cortical components, as examined by equilibrium sedimentation, directly reflects the physiologic interface between the membrane and cytoskeleton of the neutrophil populations studied and thus offers, for the first time, substantial insight into the structural and biochemical changes occurring in plasma membranes during the initial stages of blood-neutrophil activation. Unexpectedly, the predominantly cytoplasmic, membrane-unassociated organization of primary cortical components, fodrin and F-actin, in dextran-unprimed neutrophils suggests that circulating, nonactivated blood-neutrophils have an underdeveloped or 'minimal' cortex. Although our immunocytochemical and biochemical studies failed to detect a membrane cortex in this population, some system of structural support must exist to protect circulating leukocytes from the mechanical stress they encounter in the vasculature. We expect that this structural support is conferred by lower affinity membrane anchorage to the cellular actin cytoskeleton afforded by regulation of the phosphorylation state of the membrane or membrane skeletal components (Cai et al., 2005;Heredia et al., 2006;Hai and Gu, 2006), sequestration of anchorage sites in membrane organelles such as the SV (Perkins, 1984;Turrini et al., 2003), sequestration of cytoskeletal proteins in inhibitory complexes with other proteins and/or membrane lipids , (Hilpela et al., 2004;Li et al., 2005;Chen and Li, 2005) or other unexamined interactions (e.g. tubulin cytoskeleton, intermediate filaments etc.)

The conversion of neutrophils from nonactivated to functionally primed cellular states was accompanied by a structural maturation of the cortex in terms of a markedly enhanced fodrin and F-actin content. The redistribution of fodrin to the cortex of gelatin-primed neutrophils was coincident with the surface translocation of intracellular CD45.

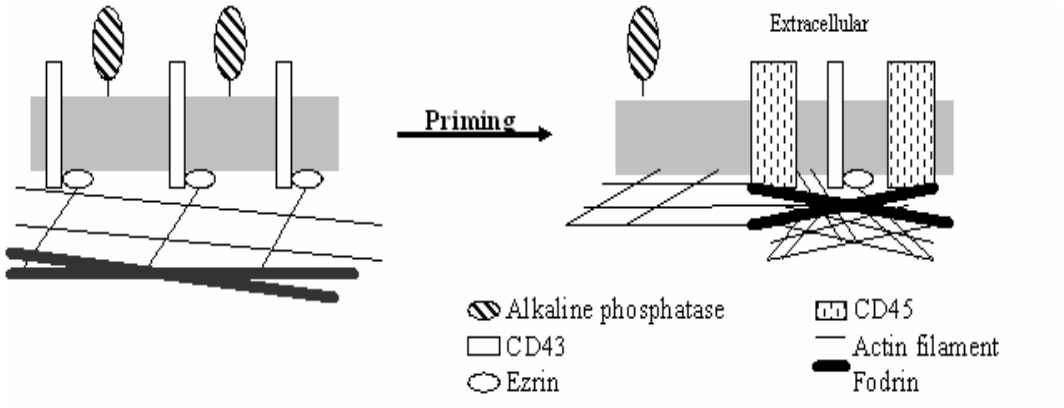


Figure 13. Model of plasma membrane structure before (left) and after (right) priming. Prior to neutrophil priming, the plasma membrane-bound proteins, AP (a GPI-linked protein), CD43, and the peripheral CD43-associated ERM protein, ezrin, are uniformly distributed on the cell surface. Furthermore, predominantly intracellular distributions of F-actin and fodrin contribute to a minimal, underdeveloped cortical structure in non-primed neutrophils. Following neutrophil priming and the mobilization of secretory vesicles, cellular fodrin moves from an intracellular distribution to the cytoplasmic face of the plasma membrane. Cortical integration of cellular fodrin is accompanied by the translocation of intracellular CD45 to the cell surface. The resulting ~3-fold increase of CD45 expression in plasma membranes coincides with the dynamic reorganization of plasma membrane structure in primed neutrophils. Two major domains can be clearly distinguished in post nuclear supernatants following equilibrium sedimentation on sucrose density gradients. These domains include an alkaline phosphatase-enriched CD43/CD45/fodrin-poor domain at ~29% sucrose and an alkaline phosphatase-poor CD45/fodrin-enriched domain at ~34% sucrose. The latter domain is also enriched in F-actin, CD43 and ezrin assembled into a functional complex in leukocyte plasma membranes. This structure is analogous to the glycophorin C-protein 4.1-F-actin complexes that anchor erythrocyte plasma membranes to the cortical skeleton.

Indeed, cortical development in gelatin-primed neutrophils was further accompanied by the establishment of a detectable membrane microdomain structure, which could be defined on the basis of differences in the density and composition of plasma membrane-derived cavitation fragments, and we infer, the lateral organization of surface AP relative to components of the F-actin/fodrin membrane–skeleton complexes (Figure 13). Significantly, this observed structural differentiation of primed plasma membranes correlates with a substantially enhanced basal adherence capacity (Figure 1A) and superoxide generation in response to fMLF (Figure 1B).

The structural compartmentalization of plasma membranes is a fundamental feature eukaryotic cell function, and is particularly apparent in the activation and differentiation of mammalian cells (Simson et al., 1998;Kusumi et al., 1999;Ritchie and Kusumi, 2004). Mechanisms by which skeletal structure influence such processes are well established and include the (i) direct functional modulation of cytosolic and transmembrane effector proteins as well as the (ii) assembly and organization of multi-component signaling complexes. This might suggest a direct cause/effect-association between the dramatic assembly of cortical structure observed in gelatin-primed neutrophils and their functional priming. Consistent with this possibility is our additional finding that dextran-unprimed neutrophils, which lacked a detectable cortical structure, were found to be minimally responsive.

Lastly, because gelatin-primed neutrophils derive from exposure to a milieu rich in proinflammatory bacterial and cellular products as well as blood-erythrocytes, leukocytes and platelets, we contend that this complex mixture of mediators and blood

cells to a reasonable degree mimics conditions present within the inflamed vasculature adjacent tissue sites of bacterial infection. As such, the gelatin method of preparing neutrophils may approximate the pathophysiological conditions responsible for the initial priming and recruitment of blood neutrophils *in vivo*. Other advantages of studying gelatin-primed neutrophils technique include facilitation of the study primed neutrophils without a extra priming protocol and subsequently allowing rapid isolation of density-purified plasma membranes that are free from contaminating SV.

In summary, results from this study demonstrate that (i) unprimed, nonadherent blood-neutrophil populations possess an underdeveloped/minimal cortex and that (ii) the functional conversion to primed blood-neutrophils is associated with the maturation of cortical structure as well as a dramatic lateral reorganization of cortical components and anchorage sites relative to surface AP. This structurally reorganized primed plasma membrane occurs in the absence of substrate adhesion, phagocytosis, and directed motility and thus highlights the currently underappreciated intrinsic potential of neutrophils to undergo sustained, substantial changes in cortical remodeling in the absence of the classical polarizing stimuli.

LITERATURE CITED

Adamus G, Zam ZS, Arendt A, Palczewski K, McDowell JH, and Hargrave PA. 1991. Anti-rhodopsin monoclonal antibodies of defined specificity: characterization and application. Vision Res 31:17-31.

Baehner RL and Boxer LA. 1979. Role of membrane vitamin E and cytoplasmic glutathione in the regulation of phagocytic functions of neutrophils and monocytes. Am J Pediatr Hematol Oncol 1:71-76.

Bengtsson T, Jaconi ME, Gustafson M, Magnusson KE, Theler JM, Lew DP, and Stendahl O. 1993. Actin dynamics in human neutrophils during adhesion and phagocytosis is controlled by changes in intracellular free calcium. Eur J Cell Biol 62:49-58.

Borregaard N. 1996. Current concepts about neutrophil granule physiology. Curr Opin Hematol 3:11-18.

Borregaard N, Christensen L, Bejerrum OW, Birgens HS, and Clemmensen I. 1990. Identification of a highly mobilizable subset of human neutrophil intracellular vesicles that contains tetranectin and latent alkaline phosphatase. J Clin Invest 85:408-416.

Borregaard N, Kjeldsen L, Rygaard K, Bastholm L, Nielsen MH, Sengelov H, Bjerrum OW, and Johnsen AH. 1992. Stimulus-dependent secretion of plasma proteins from human neutrophils. J Clin Invest 90:86-96.

Borregaard N, Kjeldsen L, and Sengelov H. 1993b. Mobilization of granules in neutrophils from patients with myeloproliferative disorders. Eur J Haematol 50:189-199.

Borregaard N, Kjeldsen L, Sengelov H, Diamond MS, Springer TA, Anderson HC, Kishimoto TK, and Bainton DF. 1994. Changes in subcellular localization and surface expression of L-selectin, alkaline phosphatase, and Mac-1 in human neutrophils during stimulation with inflammatory mediators. J Leukoc Biol 56:80-87.

Borregaard N, Lollike K, Kjeldsen L, Sengelov H, Bastholm L, Nielsen MH, and Bainton DF. 1993a. Human neutrophil granules and secretory vesicles. Eur J Haematol 51:187-198.

Borregaard N, Miller LJ, and Springer TA. 1987. Chemoattractant-regulated mobilization of a novel intracellular compartment in human neutrophils. Science 237:1204-1206.

Bourguignon LY, Suchard SJ, Nagpal ML, and Glenney JR, Jr. 1985. A T-lymphoma transmembrane glycoprotein (gp180) is linked to the cytoskeletal protein, fodrin. J Cell Biol 101:477-487.

Brown GE, Stewart MQ, Bissonnette SA, Elia AE, Wilker E, and Yaffe MB. 2004. Distinct ligand-dependent roles for p38 MAPK in priming and activation of the neutrophil NADPH oxidase. J Biol Chem 279:27059-27068.

Burritt JB, DeLeo FR, McDonald CL, Prigge JR, Dinauer MC, Nakamura M, Nauseef WM, and Jesaitis AJ. 2001. Phage display epitope mapping of human neutrophil flavocytochrome b558. Identification of two juxtaposed extracellular domains. J Biol Chem 276:2053-2061.

Cadwallader KA, Uddin M, Condliffe AM, Cowburn AS, White JF, Skepper JN, Ktistakis NT, and Chilvers ER. 2004. Effect of priming on activation and localization of phospholipase D-1 in human neutrophils. Eur J Biochem 271:2755-2764.

Cai L, Holoweckyj N, Schaller MD, and Bear JE. 2005. Phosphorylation of coronin 1B by protein kinase C regulates interaction with Arp2/3 and cell motility. J Biol Chem 280:31913-31923.

Calafat J, Kuijpers TW, Janssen H, Borregaard N, Verhoeven AJ, and Roos D. 1993. Evidence for small intracellular vesicles in human blood phagocytes containing cytochrome b558 and the adhesion molecule CD11b/CD18. Blood 81:3122-3129.

Cano ML, Cassimeris L, Fechheimer M, and Zigmond SH. 1992. Mechanisms responsible for F-actin stabilization after lysis of polymorphonuclear leukocytes. J Cell Biol 116:1123-1134.

Cano ML, Lauffenburger DA, and Zigmond SH. 1991. Kinetic analysis of F-actin depolymerization in polymorphonuclear leukocyte lysates indicates that chemoattractant stimulation increases actin filament number without altering the filament length distribution. J Cell Biol 115:677-687.

Cassimeris L, McNeill H, and Zigmond SH. 1990. Chemoattractant-stimulated polymorphonuclear leukocytes contain two populations of actin filaments that differ in their spatial distributions and relative stabilities. J Cell Biol 110:1067-1075.

Chen Y and Li M. 2005. Interactions between CAP70 and actinfilin are important for integrity of actin cytoskeleton structures in neurons. Neuropharmacology 49:1026-1041.

Condliffe AM, Kitchen E, and Chilvers ER. 1998. Neutrophil priming: pathophysiological consequences and underlying mechanisms. Clin Sci (Lond) 94:461-471.

Dahinden C and Fehr J. 1983. Granulocyte activation by endotoxin. II. Role of granulocyte adherence, aggregation, and effect of cytochalasin B, and comparison with formylated chemotactic peptide-induced stimulation. J Immunol 130:863-868.

Dahinden C, Galanos C, and Fehr J. 1983. Granulocyte activation by endotoxin. I. Correlation between adherence and other granulocyte functions, and role of endotoxin structure on biologic activity. J Immunol 130:857-862.

Dang PM, Stensballe A, Boussetta T, Raad H, Dewas C, Kroviarski Y, Hayem G, Jensen ON, Gougerot-Pocidalo MA, and El Benna J. 2006. A specific p47 -serine phosphorylated by convergent MAPKs mediates neutrophil NADPH oxidase priming at inflammatory sites. J Clin Invest.

Faurschou M and Borregaard N. 2003. Neutrophil granules and secretory vesicles in inflammation. Microbes Infect 5:1317-1327.

Fehr J and Jacob HS. 1977. In vitro granulocyte adherence and in vivo margination: two associated complement-dependent functions. Studies based on the acute neutropenia of filtration leukophoresis. J Exp Med 146:641-652.

Gabig TG. 1980. Leukocyte abnormalities. Med Clin North Am 64:647-666.

Ginis I and Tauber AI. 1990. Activation mechanisms of adherent human neutrophils. Blood 76:1233-1239.

Guichard C, Pedruzzi E, Dewas C, Fay M, Pouzet C, Bens M, Vandewalle A, Ogier-Denis E, Gougerot-Pocidalo MA, and Elbim C. 2005. Interleukin-8-induced priming of neutrophil oxidative burst requires sequential recruitment of NADPH oxidase components into lipid rafts. J Biol Chem 280:37021-37032.

Hai CM and Gu Z. 2006. Caldesmon phosphorylation in actin cytoskeletal remodeling. Eur J Cell Biol 85:305-309.

Haslett C, Worthen GS, Giclas PC, Morrison DC, Henson JE, and Henson PM. 1987. The pulmonary vascular sequestration of neutrophils in endotoxemia is initiated by an effect of endotoxin on the neutrophil in the rabbit. Am Rev Respir Dis 136:9-18.

Henson PM and Oades ZG. 1973. Enhancement of immunologically induced granule exocytosis from neutrophils by cytochalasin B. J Immunol 110:290-293.

Henson PM and Oades ZG. 1975. Stimulation of human neutrophils by soluble and insoluble immunoglobulin aggregates. Secretion of granule constituents and increased oxidation of glucose. J Clin Invest 56:1053-1061.

Heredia L, Helguera P, de Olmos S, Kedikian G, Sola VF, LaFerla F, Staufenbiel M, de Olmos J, Busciglio J, Caceres A, and Lorenzo A. 2006. Phosphorylation of actin-depolymerizing factor/cofilin by LIM-kinase mediates amyloid beta-induced degeneration: a potential mechanism of neuronal dystrophy in Alzheimer's disease. J Neurosci 26:6533-6542.

Hilpela P, Vartiainen MK, and Lappalainen P. 2004. Regulation of the actin cytoskeleton by PI(4,5)P2 and PI(3,4,5)P3. Curr Top Microbiol Immunol 282:117-163.

Hyslop PA, Oades ZG, Jesaitis AJ, Painter RG, Cochrane CG, and Sklar LA. 1984. Evidence for N-formyl chemotactic peptide-stimulated GTPase activity in human neutrophil homogenates. FEBS Lett 166:165-169.

Jesaitis AJ, Bokoch GM, Tolley JO, and Allen RA. 1988. Lateral segregation of neutrophil chemotactic receptors into a. J Cell Biol 107:921-928.

Jesaitis AJ, Erickson RW, Klotz KN, Bommakanti RK, and Siemsen DW. 1993. Functional molecular complexes of human N-formyl chemoattractant receptors and actin. J Immunol 151:5653-5665.

Jesaitis AJ and Klotz KN. 1993. Cytoskeletal regulation of chemotactic receptors: molecular complexation of N-formyl peptide receptors with G proteins and actin. Eur J Haematol 51:288-293.

Jesaitis AJ, Tolley JO, and Allen RA. 1986. Receptor-cytoskeleton interactions and membrane traffic may regulate chemoattractant-induced superoxide production in human granulocytes. J Biol Chem 261:13662-13669.

Jesaitis AJ, Tolley JO, Bokoch GM, and Allen RA. 1989. Regulation of chemoattractant receptor interaction with transducing proteins by organizational control in the plasma membrane of human neutrophils. J Cell Biol 109:2783-2790.

Kitchen E, Rossi AG, Condliffe AM, Haslett C, and Chilvers ER. 1996. Demonstration of reversible priming of human neutrophils using platelet-activating factor. Blood 88:4330-4337.

Klotz KN and Jesaitis AJ. 1994. Neutrophil chemoattractant receptors and the membrane skeleton. Bioessays 16:193-198.

Kobayashi SD, Voyich JM, Burlak C, and DeLeo FR. 2005. Neutrophils in the innate immune response. Arch Immunol Ther Exp (Warsz) 53:505-517.

Kusumi A, Suzuki K, and Koyasako K. 1999. Mobility and cytoskeletal interactions of cell adhesion receptors. Curr Opin Cell Biol 11:582-590.

Kvietys PR and Sandig M. 2001. Neutrophil diapedesis: paracellular or transcellular? News Physiol Sci 16:15-19.

Li J, Dai Z, Jana D, Callaway DJ, and Bu Z. 2005. Ezrin controls the macromolecular complexes formed between an adapter protein Na+/H+ exchanger regulatory factor and the cystic fibrosis transmembrane conductance regulator. J Biol Chem 280:37634-37643.

Liu JJ, Song CW, Yue Y, Duan CG, Yang J, He T, and He YZ. 2005. Quercetin inhibits LPS-induced delay in spontaneous apoptosis and activation of neutrophils. Inflamm Res 54:500-507.

Lokeshwar VB and Bourguignon LY. 1992. Tyrosine phosphatase activity of lymphoma CD45 (GP180) is regulated by a direct interaction with the cytoskeleton. J Biol Chem 267:21551-21557.

MacKinnon AC, Buckley A, Chilvers ER, Rossi AG, Haslett C, and Sethi T. 2002. Sphingosine kinase: a point of convergence in the action of diverse neutrophil priming agents. J Immunol 169:6394-6400.

McPhail LC, Clayton CC, and Snyderman R. 1984. The NADPH oxidase of human polymorphonuclear leukocytes. Evidence for regulation by multiple signals. J Biol Chem 259:5768-5775.

Meldrum DR, Cleveland JC, Jr., Moore EE, Partrick DA, Banerjee A, and Harken AH. 1997. Adaptive and maladaptive mechanisms of cellular priming. Ann Surg 226:587-598.

Morgan CP, Sengelov H, Whatmore J, Borregaard N, and Cockcroft S. 1997. ADP-ribosylation-factor-regulated phospholipase D activity localizes to secretory vesicles and mobilizes to the plasma membrane following N-formylmethionyl-leucyl-phenylalanine stimulation of human neutrophils. Biochem J 325 (Pt 3):581-585.

Mukherjee G, Quinn MT, Linner JG, and Jesaitis AJ. 1994. Remodeling of the plasma membrane after stimulation of neutrophils with f-Met-Leu-Phe and dihydrocytochalasin B: identification of membrane subdomains containing NADPH oxidase activity. J Leukoc Biol 55:685-694.

Niwa M, Hotta K, Kanamori Y, Kumada M, Hirota M, Kozawa O, and Fujimoto S. 2004. p38 MAPK associated with stereoselective priming by grepafloxacin on O2- production in neutrophils. Free Radic Biol Med 36:1259-1269.

Omann GM, Swann WN, Oades ZG, Parkos CA, Jesaitis AJ, and Sklar LA. 1987. N-formylpeptide-receptor dynamics, cytoskeletal activation, and intracellular calcium response in human neutrophil cytoplasts. J Immunol 139:3447-3455.

Park EY, Smith MJ, Stropp ES, Snapp KR, DiVietro JA, Walker WF, Schmidtke DW, Diamond SL, and Lawrence MB. 2002. Comparison of PSGL-1 microbead and neutrophil rolling: microvillus elongation stabilizes P-selectin bond clusters. Biophys J 82:1835-1847.

Perkins ME. 1984. Receptor mediated endocytosis of the malarial parasite by erythrocytes. Prog Clin Biol Res 165:361-376.

Philips MR, Abramson SB, Kolasinski SL, Haines KA, Weissmann G, and Rosenfeld MG. 1991. Low molecular weight GTP-binding proteins in human neutrophil granule membranes. J Biol Chem 266:1289-1298.

Quinn MT, Parkos CA, and Jesaitis AJ. 1989. The lateral organization of components of the membrane skeleton and superoxide generation in the plasma membrane of stimulated human neutrophils. Biochim Biophys Acta 987:83-94.

Ritchie K and Kusumi A. 2004. Role of the membrane skeleton in creation of microdomains. Subcell Biochem 37:233-245.

Ryder MI, Weinreb RN, and Niederman R. 1984. The organization of actin filaments in human polymorphonuclear leukocytes. Anat Rec 209:7-20.

Sawyer DW, Donowitz GR, and Mandell GL. 1989. Polymorphonuclear neutrophils: an effective antimicrobial force. Rev Infect Dis 11 Suppl 7:S1532-S1544.

Seely AJ, Pascual JL, and Christou NV. 2003. Science review: Cell membrane expression (connectivity) regulates neutrophil delivery, function and clearance. Crit Care 7:291-307.

Sengelov H, Boulay F, Kjeldsen L, and Borregaard N. 1994. Subcellular localization and translocation of the receptor for N-formylmethionyl-leucyl-phenylalanine in human neutrophils. Biochem J 299 (Pt 2):473-479.

Sengelov H, Follin P, Kjeldsen L, Lollike K, Dahlgren C, and Borregaard N. 1995. Mobilization of granules and secretory vesicles during in vivo exudation of human neutrophils. J Immunol 154:4157-4165.

Sengelov H, Kjeldsen L, and Borregaard N. 1993a. Control of exocytosis in early neutrophil activation. J Immunol 150:1535-1543.

Sengelov H, Kjeldsen L, Diamond MS, Springer TA, and Borregaard N. 1993b. Subcellular localization and dynamics of Mac-1 (alpha m beta 2) in human neutrophils. J Clin Invest 92:1467-1476.

Sengelov H, Nielsen MH, and Borregaard N. 1992. Separation of human neutrophil plasma membrane from intracellular vesicles containing alkaline phosphatase and NADPH oxidase activity by free flow electrophoresis. J Biol Chem 267:14912-14917.

Serrador JM, Nieto M, Alonso-Lebrero JL, del Pozo MA, Calvo J, Furthmayr H, Schwartz-Albiez R, Lozano F, Gonzalez-Amaro R, Sanchez-Mateos P, and Sanchez-Madrid F. 1998. CD43 interacts with moesin and ezrin and regulates its redistribution to the uropods of T lymphocytes at the cell-cell contacts. Blood 91:4632-4644.

Shao JY, Ting-Beall HP, and Hochmuth RM. 1998. Static and dynamic lengths of neutrophil microvilli. Proc Natl Acad Sci U S A 95:6797-6802.

Simson R, Yang B, Moore SE, Doherty P, Walsh FS, and Jacobson KA. 1998. Structural mosaicism on the submicron scale in the plasma membrane. Biophys J 74:297-308.

Sklar LA, Finney DA, Oades ZG, Jesaitis AJ, Painter RG, and Cochrane CG. 1984. The dynamics of ligand-receptor interactions. Real-time analyses of association, dissociation, and internalization of an N-formyl peptide and its receptors on the human neutrophil. J Biol Chem 259:5661-5669.

Sklar LA and Oades ZG. 1985. Signal transduction and ligand-receptor dynamics in the neutrophil. Ca2+ modulation and restoration. J Biol Chem 260:11468-11475.

Sklar LA, Oades ZG, Jesaitis AJ, Painter RG, and Cochrane CG. 1981. Fluoresceinated chemotactic peptide and high-affinity antifluorescein antibody as a probe of the temporal characteristics of neutrophil stimulation. Proc Natl Acad Sci U S A 78:7540-7544.

Stefanidakis M, Ruohtula T, Borregaard N, Gahmberg CG, and Koivunen E. 2004. Intracellular and cell surface localization of a complex between alphaMbeta2 integrin and promatrix metalloproteinase-9 progelatinase in neutrophils. J Immunol 172:7060-7068.

Stie J and Jesaitis A.J. 2004. Preparation of Secretory Vesicle-Free Plasma Membranes by Isopycnic Sucrose Gradeint Fractionation of Neutrophils Purified by the Gelatin Method. Cytotechnology 46: 109-122.
Ref Type: Generic

Sullivan JA and Mandell GL. 1983. Motility of human polymorphonuclear neutrophils: microscopic analysis of substrate adhesion and distribution of F-actin. Cell Motil 3:31-46.

Swain SD, Rohn TT, and Quinn MT. 2002. Neutrophil priming in host defense: role of oxidants as priming agents. Antioxid Redox Signal 4:69-83.

Tolley JO, Omann GM, and Jesaitis AJ. 1987. A high-yield, high-purity elutriation method for preparing human granulocytes demonstrating enhanced experimental lifetimes. J Leukoc Biol 42:43-50.

Turrini F, Giribaldi G, Carta F, Mannu F, and Arese P. 2003. Mechanisms of band 3 oxidation and clustering in the phagocytosis of Plasmodium falciparum-infected erythrocytes. Redox Rep 8:300-303.

Valerius NH, Stendahl O, Hartwig JH, and Stossel TP. 1981. Distribution of actin-binding protein and myosin in polymorphonuclear leukocytes during locomotion and phagocytosis. Cell 24:195-202.

Vosbeck K, Tobias P, Mueller H, Allen RA, Arfors KE, Ulevitch RJ, and Sklar LA. 1990. Priming of polymorphonuclear granulocytes by lipopolysaccharides and its

complexes with lipopolysaccharide binding protein and high density lipoprotein. J Leukoc Biol 47:97-104.

Watts RG and Howard TH. 1992. Evidence for a gelsolin-rich, labile F-actin pool in human polymorphonuclear leukocytes. Cell Motil Cytoskeleton 21:25-37.

Watts RG and Howard TH. 1994. Role of tropomyosin, alpha-actinin, and actin binding protein 280 in stabilizing Triton insoluble F-actin in basal and chemotactic factor activated neutrophils. Cell Motil Cytoskeleton 28:155-164.

Worthen GS, Haslett C, Rees AJ, Gumbay RS, Henson JE, and Henson PM. 1987. Neutrophil-mediated pulmonary vascular injury. Synergistic effect of trace amounts of lipopolysaccharide and neutrophil stimuli on vascular permeability and neutrophil sequestration in the lung. Am Rev Respir Dis 136:19-28.

Worthen GS, Haslett C, Smedly LA, Rees AJ, Gumbay RS, Henson JE, and Henson PM. 1986. Lung vascular injury induced by chemotactic factors: enhancement by bacterial endotoxins. Fed Proc 45:7-12.

Yamashiro S, Kamohara H, Wang JM, Yang D, Gong WH, and Yoshimura T. 2001. Phenotypic and functional change of cytokine-activated neutrophils: inflammatory neutrophils are heterogeneous and enhance adaptive immune responses. J Leukoc Biol 69:698-704.

Yeung T, Georges PC, Flanagan LA, Marg B, Ortiz M, Funaki M, Zahir N, Ming W, Weaver V, and Janmey PA. 2005. Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion. Cell Motil Cytoskeleton 60:24-34.

Yonemura S, Hirao M, Doi Y, Takahashi N, Kondo T, Tsukita S, and Tsukita S. 1998. Ezrin/radixin/moesin (ERM) proteins bind to a positively charged amino acid cluster in the juxta-membrane cytoplasmic domain of CD44, CD43, and ICAM-2. J Cell Biol 140:885-895.

Yonemura S, Nagafuchi A, Sato N, and Tsukita S. 1993. Concentration of an integral membrane protein, CD43 (leukosialin, sialophorin), in the cleavage furrow through the interaction of its cytoplasmic domain with actin-based cytoskeletons. J Cell Biol 120:437-449.

Young SK, Worthen GS, Haslett C, Tonnesen MG, and Henson PM. 1990. Interaction between chemoattractants and bacterial lipopolysaccharide in the induction and enhancement of neutrophil adhesion. Am J Respir Cell Mol Biol 2:523-532.

Zhelev DV and Hochmuth RM. 1995. Mechanically stimulated cytoskeleton rearrangement and cortical contraction in human neutrophils. Biophys J 68:2004-2014.

CHAPTER 4

LOCALIZATION OF HCAP TO THE CELL SURFACE OF HUMAN NEUTROPHILS USING TWO NOVEL HCAP-SPECIFIC MONOCLONAL ANTIBODIES

Abstract

We describe the characterization of two Mabs developed against neutrophil cellular membranes. Both recognize a single ~16 kD band identified by N-terminal sequence analysis as the human cathelicidin, hCap. Mass analysis of the trypsin-digested antigen by MALDI mass spectrometry gave respective sequence coverages of 55% and 65% for pro/cathelin and cationic peptide domains. The ~16 kD antigen also co-distributed with lactoferrin in density fractionated post-nuclear fractions from resting neutrophils, in agreement with the specific granule localization of neutrophil-hCap. Phage display peptide library analysis suggested that both Mabs recognize a five amino acid sequence located within the C-terminal region of the pro/cathelin domain. Subsequent immunopurification of antigen from degranulated neutrophil supernatants yielded a 14 kD fragment identified as the pro/cathelin domain by mass spectrometry. FACs analysis of fMLF-activated neutrophil populations suggested an unexpected association of hCap with activated plasma membranes that was substantially enhanced on intact surfaces of fully degranulated neutrophils. Subcellular fractionation studies employing fMLF-stimulated neutrophil populations showed a bimodal distribution of the ~16 kD antigen within gradient regions enriched with plasma membranes and specific granules. Additional immunoprecipitation studies employing purified plasma membranes from

fMLF-stimulated and fully degranulated cells followed by MALDI mass spectrometry confirmed the presence of the ~16 kD hCap. The nature of hCap surface interaction proved to be a high affinity/hydrophobic association resistant to high molar salt washes and co-partitioning with a detergent-rich phase after Triton X-114 phase extraction of degranulated plasma membranes. These findings, together with the development of two hCap-specific monoclonals, offer a basis for additional investigation into the biological function of hCap.

Abbreviations

ABTS, 2,2'-azino-bis [3-ethylbenziazoline-6-sulfonic acid]; ATP, adenosine tri-phosphate; DFP, di-isopropylfluorophosphate; DPBS , Delbecco's phosphate-buffered saline; $DPBS^{4+}$, DPBS supplemented with 0.9 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1% bovine serum albumin and 0.1% dextrose; fMLF, N-formyl-methionyl-leucyl-phenylalanine; EDTA, ethylene-diaminetetraceticacid-disodium-salt-dihydrate; HEPES, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; ME, 2-mercapto-ethanol; PMSF, phenyl-methylsulphonyl-fluoride; PVDF, polyvinylidene fluoride membranes.

Introduction

Over the past 30 years, numerous, structurally diverse cationic peptides exhibiting broad-spectrum antimicrobial activity have been described in plants and animals (Zasloff, 2002). Though subject to species-specific patterns of expression and differences in post-translational modification and regulation, all peptide families

described thus far target and disintegrate microbial membranes by charge-based interactions and bilayer-penetrating amphipathic associations, respectively (Tossi et al., 2000;Shai, 2002). Defensins and cathelicidins, two of the main peptide families expressed in humans, are distinguished by differences in gene structure and amino acid composition. Defensins are a multi-gene family distinguished by relatively arginine-rich sequences with six conserved disulfide-bonding cysteine residues and are fully active following translation (Ganz et al., 1985), whereas cathelicidins consist of a single-gene product in humans that is translated as a precursor molecule made functionally active by protease cleavage after secretion (Zanetti et al., 1995;Lehrer and Ganz, 2002). Thus, the distinctive gene structure of mammalian cathelicidins allows for coupling of highly conserved N-terminal signal- and pro-sequences to C-terminal residues encoding the active peptide and subsequently determines the intracellular processing, secretion and activation requirements of the holo-protein.

The extent to which the human cathelicidin, hCap, contributes to the complex signaling and regulatory networks that mediate the microbicidal function of the innate immune response is still being realized. Recent reports examining the impact of cathelicidin expression on infection and immunity indicates a primary role for this protein in the containment and eradication of infectious disease. Results from one study involving patients with Kostmann disease, a severe congenital neutropenia, implicated a marked deficiency in hCap expression in the etiology of disease. Specifically, investigators correlated the presentation of recurrent infections and periodontal disease associated with this disorder with an additional deficiency in hCap expression in plasma,

saliva and neutrophils. Importantly, patient neutrophils were otherwise normal in ability to activate and produce superoxide (Putsep et al., 2002). Restoration of patient-hCap expression levels, but not blood-neutrophil levels, resulted in disease remediation. Another study found hCap expression to be differentially regulated in Th1- versus Th2-dominated skin disease with low expression levels observed in Th2 responses correlating with disease susceptibility (Ong et al., 2002). Additional gene transfer studies found hCap overexpression in mouse models of local and systemic disease resulted in lower bacterial load and mortality, respectively (Bals et al., 1999a), and gene transfer of hCap in a cystic fibrosis xenograft model restored intrinsic deficiencies in microbicidal activity to normal levels (Bals et al., 1999b). Finally, expression of CRAMP, a mouse cathelicidin that is similar in peptide structure, activity and distribution to hCap, was required for bacterial clearance following cutaneous infection by Group A Streptococcus (Nizet et al., 2001).

The antibiotic potential of hCap is classically attributed to the direct antimicrobial actions of the protease-cleaved C-terminal fragment, LL-37, a 37 amino acid peptide flanked by two N-terminal leucine residues (Tomasinsig and Zanetti, 2005). However, recent work has demonstrated an unexpected biological complexity and functional heterogeneity associated with hCap activity. For example, hCap can be specifically targeted for cleavage by multiple proteases in a tissue-specific manner. While hCap cleavage by protease 3, a serine protease enriched in neutrophil azurophilic granules, within neutrophil-rich, infected tissues yields LL-37 (Sorensen et al., 2001), hCap cleavage by either gastricsin (Sorensen et al., 2003), a serine protease enriched in the

prostate, or proteases present in human sweat (Murakami et al., 2004), yields alternative cleavage products with characteristic properties. For instance, ALL-38, the product of gastricsin cleavage, has an additional N-terminal alanine residue but is otherwise similar in activity to LL-37, whereas the peptides resulting from cleavage by sweat proteases have functional properties distinct from those of LL-37.

Beyond its core properties of microbicidal (Oren et al., 1999) and LPS-sequestering activity (Kirikae et al., 1998), LL-37 is implicated in the regulation of innate and humoral immune responses, wound healing, and tissue repair. The capacity of LL-37 to regulate cellular and molecular networks associated with innate and humoral immune responses is at least partially due to LL-37 binding to and activation of cellular receptors, including (i) chemoattractant receptor FPRL-1, expressed by human neutrophils, monocytes, macrophages, T cells and mast cells (De et al., 2000;Niyonsaba et al., 2002); (ii) P2X7 receptor expressed on LPS-primed human monocytes (Elssner et al., 2004); and, by a process of transactivation, the EGFR of airway epithelial cells (Tjabringa et al., 2003). These studies report functional LL-37-cellular receptor interactions within range of the LL-37 concentration expected at sites of inflammation (5-50 ug/ml). LL-37-cellular interactions can also alter plasma membrane-bound receptor expression profile of human macrophages (Scott et al., 2002), modulate dendritic cell maturation and, consequently, the nature of T-cell immunity (Davidson et al., 2004), and induce mast cell degranulation (Niyonsaba et al., 2001). Along other lines of investigation, studies examining wound healing in an *ex vivo* model of organ-cultured human skin have shown that hCap-depletion inhibits the re-epithelialization of skin wounds

(Heilborn et al., 2003). Similar studies employing *in vitro* cultured endothelial cells found exposure to LL-37 stimulated cellular proliferation and formation of vessel-like structures (Koczulla et al., 2003), suggesting a role for LL-37 in angiogenesis. Additional findings that hCap is widely expressed in leukocytes, including neutrophils (Cowland et al., 1995), monocytes, T cells and mast cells (Oren et al., 1999;Di Nardo et al., 2003), as well as in keratinocytes (Frohm et al., 1997) and various columnar and squamous epithelia (Bals et al., 1998;Frohm et al., 1999;Malm et al., 2000;Islam et al., 2001), further suggest the global importance and versatility of hCap function in diverse molecular and cellular processes.

The current study describes the characterization of two hCap-specific monoclonal antibodies and novel patterns hCap expression on plasma membranes of resting, fMLF-activated and fully degranulated human neutrophils. Structural analysis of epitope binding sites using a phage library initially identified an anti-hCap specificity with antibody recognition sites located within the C-terminal region of the pro/cathelin domain. The immuno-purified protein corresponded to a ~16 kD protein by SDS-PAGE analysis, similar to the molecular mass expected for the ~18 kD native hCap. Amino acid sequence analysis using MALDI mass spectrometry analysis of immuno-purified, trypsin-digested antigen confirmed hCap as the antigen bound by both monoclonal antibodies. Subsequent localization studies using isopycnic sucrose density sedimentation of nitrogen-cavitated cellular lysates from purified populations of human neutrophils indicated co-enrichment of antigen with lactoferrin, a marker protein for specific

granules. A more detailed characterization of antigen localization further indicated an activation-dependent association of hCap with the cell surface of intact human neutrophil populations. Thus, in addition to the identification and characterization of two hCap-specific monoclonal antibodies, which should prove useful tools in the field of hCap research, the novel localization properties of this protein reported in this study may afford further insight into the complex biology of this protein.

Materials and Methods

Materials

Ficoll-Hypaque, dextran T-500 and CNBr activated sepharose beads were purchased from Amersham Biosciences (Piscataway, NJ); HRP-goat anti-mouse and HRP-goat anti-rabbit IgG antibody from Bio-Rad Laboratories (Hercules, CA); bovine liver superoxide dismutase, diisopropyl fluorophosphate (DFP) and phenyl-methylsulphonyl-fluoride (PMSF) from Calbiochem (La Jolla, CA); gelatin and dextrose from Fisher Scientific (Pittsburg, PA); water-for-injection (WFI)-grade distilled water from HyClone (Logan, UT); sucrose and rabbit anti-lactoferrin antisera from ICN Biomedicals (Aurora, OH); polyvinylidene-difluoride immobilon (PVDF) membranes from Millipore (Bedford, MA); goat serum from MP Biomedicals (Irvine, CA); Supersignal chemiluminescent detection kit from Pierce Biotechnology (Rockford, IL); N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES) and dodecyl maltoside (DDM) were from Sigma Chemical Co. (St Louis, MO). All additional materials were purchased from Sigma unless otherwise indicated.

Monoclonal Antibodies

Monoclonal antibody H7 and N9 were produced using standard hybridoma technology as previously described (Burritt et al., 1995;Burritt et al., 2003). Hybridoma clones producing anti-Cyt b mAbs were cloned twice by limiting dilution and grown in RPMI medium containing 5% fetal calf serum. Antibody was collected on Gammabind Sepharose beads (Pharmacia, Piscataway, NJ), and eluted with 100 mM glycine, 150 mM NaCl, pH 2.5 into neutralization buffer and dialyzed against 150 mM NaCl, 10 mM Na2HPO4, pH 7.4.

N-Terminal Sequence Analysis

Antigen was immunopurified using H7-coupled beads as described below. Bead-eluate was separated by SDS-PAGE. After minimal staining with Coomassie blue, a single ~16 kD protein was visualized. Following electrotransfer onto PVDF membrane, the band was removed and sent off to Harvard Microchem for sequence analysis.

Phage Display Analysis

One point two milligrams of mAb H7 and N9 were individually subjected to epitope mapping as previously described, by selecting peptide sequences that mimic the Cap 18 epitope from the J404 nonapeptide library. The J404 library is a collection of 5 x 108 unique M13-based bacteriophage members, in which each clone of the library displays up to five copies of a unique nine-residue peptide displayed at the amino terminus of the pIII capsid protein and contains the nucleotide segment encoding the unique peptide within the genome. Three sequential rounds of selection and amplification of phage-display peptide

library clones were carried out. For the initial round of selection, 0.4 mg of antibody was allowed to react with 1012 plaque forming units overnight at 4° C, then antibody was captured on 0.1 ml of the Gammabind Sepharose matrix for one hour at 25° C. Following extensive washing of the matrix to remove unbound phage clones, adherent phage displaying immunoreactive peptides were eluted in low pH, then amplified in host K91 cells as described (Burritt et al., 1996). After the sequential rounds of selection and amplification, the sequences of displayed peptides were deduced by nucleotide sequence analysis of the isolated clones as described (Smith and Scott, 1993).

Blood-Neutrophil Isolation

Neutrophils were isolated either by gelatin-based (Henson and Oades, 1975) or dextran-based (Borregaard et al., 1990) procedures with minor modifications as previously described (Stie and Jesaitis, 2004). Briefly, regarding gelatin-preparation, freshly drawn blood from human donors was sedimented at 260 xg for 20 min (25°C), the plasma removed and cell pellets resuspended in a pre-warmed solution of 150 mM NaCl and 2% gelatin followed by 30 min incubation (37°C). Leukocyte-enriched supernatants were isolated and the cellular fraction pelleted at 360 xg for 8 min (25°C) followed by resuspension in ice cold NH_4Cl buffer (155 mM NH_4Cl, 9.4 mM $NaHCO_3$, pH 7.4, and 130 µM EDTA) for 5 min (4°C), with occasional agitation, to facilitate red cell lysis. Cells were then washed and resuspended in $DPBS^{4+}$ (DPBS, pH 7.4, supplemented with 0.1% BSA and 0.1% dextrose), treated with 3 mM DFP for 15 min (4°C), washed in $DPBS^{4+}$, and either immediately resuspended in cavitation buffer (0.34M sucrose, 10mM

HEPES, pH 7.4, 1 mM EDTA, 0.1 mM $MgCl_2$, 1 mM ATP, 0.1 mM PMSF, pH 7.4, and protease inhibitor cocktail) or stimulated with 1 μM fMLF (see below) prior to resuspension and nitrogen cavitation at 450 psi for 15 min (4°C). Most of the experiments described in this paper employ gelatin-prepared neutrophil populations. However, due to the potential for cellular activation (and thus partial degranulation) during exposure to gelatin (Stie and Jesaitis, 2004), neutrophil populations used for the experiments described in figures 3 and 4 were conducted using dextran-prepared populations to insure that the results obtained accurately reflected a non-activated cellular state where indicated.

For the dextran technique, freshly drawn blood from human donors was mixed with an equivalent volume of saline with 3% dextran, incubated 30 min (25°C) followed by sedimentation of the leukocyte-enriched supernatants at 740 xg for 10 min (25°C). Pellets were resuspended in 35 ml saline (25°C) and underlaid with 10 ml Ficoll-Hypaque and sedimented at 415 xg for 25 min (25°C). Remaining red cells were lysed hypotonically by resuspension of pellets in ice-cold WFI-dH_2O, recovery to isotonicity followed by sedimentation at 360 xg for 8 min (4°C). Pellets were then resuspended in $DPBS^{4+}$, DFP-treated as described above, and either further stimulated with fMLF or not as indicated below prior to nitrogen cavitation. Cell counts were performed using a hemocytometer with cell aliquots obtained prior to DFP-treatment.

Subcellular Fractionation

Isopycnic Sucrose Density
Gradient Sedimentation. Purified neutrophil populations ($5x10^8$) were either stimulated with 1 μM fMLF (see below) or not stimulated prior to nitrogen cavitation. Post-nuclear fractions (5 ml) obtained after 10 min centrifugation at 890 xg (4°C) were layered onto 25 ml linear sucrose gradients and sedimented at 8.3×10^4 xg for 3.5 hr (4°C) in an SW28 Beckman rotor. Gradients were fractionated from the bottom using an Auto Densi-Flow IIC fraction collector (HBI Technologies, Phoenix, AZ) and stored at –70°C prior to use.

Differential Sedimentation. Neutrophil populations ($1x10^8$) were stimulated with 1 μM fMLF (see below), and nitrogen cavitated with post-nuclear fractions isolated as described above. Post-nuclear fractions were initially sedimented at 8,000xg for 15 minutes (4°C), using a Beckman Ti-75 rotor, to remove granules; supernatants were then sedimented at $1x10^5$ xg for 1hr to isolate plasma membrane fractions. Pelleted plasma membranes were resuspended by sonication into 700 μl PBS, pH 7.4, for immunoprecipitation assays (see below). Alternatively, cellular membranes were isolated by sedimentation of post-nuclear fractions at $1x10^5$ xg followed by resuspension in 700 μl PBS, pH 7.4, with 1% DDM for use in either immunoprecipitation assays or immediate SDS-PAGE separation and immunoblot analysis.

Flow Cytometry Analysis

After blood-isolation, human neutrophil populations were suspended $DPBS^{4+}$ supplemented with 80 µg catalase and 10,000 activity units bovine liver superoxide dismutase at 10^8 cells/ml and incubated 7 minutes (37°C) at which time 1 µM fMLF was added and incubation continued for an additional 3 minutes. Reactions were quenched with 3-fold volume of ice-cold $DPBS^{4+}$ supplemented with 2 mM EDTA, 100 µM PMSF and protease inhibitor cocktail, sedimented at 740 xg for 10 minutes (4°C), and washed 1-2 times in $DPBS^{4+}$. Degranulated neutrophil populations were obtained by adding 2 µg/ml cytochalasin B to the fMLF-stimulation buffer at the beginning of the 7 min incubation period (37°C) prior to fMLF-stimulation. Neutrophils were resuspended in $DPBS^{4+}$, distributed in 5 ml 12x75 mm Falcon round-bottom polystyrene tubes (Fisher Scientific) at 10^6 cells/tube, and labeled with 1 µg N9 or H7 for 30 minutes on ice. Reactions were quenched by adding ~50-fold volume of ice-cold $DPBS^{4+}$ followed by sedimentation at 360 xg for 8 minutes (4°C). Neutrophil populations were then labeled with secondary FITC-conjugated goat anti-mouse antibody (Bethyl Laboratories, Montgomery, TX), quenched as described above, finally resuspended in PBS, and analyzed by FACs analysis. Negative controls that were employed in these experiments include secondary labeling (in the absence of primary stain), primary labeling with irrelevant monoclonal antibody K16, and primary labeling with monoclonal antibody 44.1 specific for the cytoplasmic domain of cytochrome b_{558} transmembrane component p22. The latter control allowed for the assessment of plasma membrane integrity of

assayed populations. As negative control data were virtually identical in relative intensity, only data from the 44.1 monoclonal antibody control are included in Figure 8.

Isolation of soluble hCap and Related Cleavage Product from Intact Degranulated Neutrophils

Freshly isolated neutrophils were fMLF-stimulated in the presence of cytochalasin B as described above. Following termination of cellular activation, supernatants were isolated and concentrated through an ultrafiltration membrane with a nominal molecular weight limit of 30 kD. Filtrates were then denatured, separated by SDS-PAGE and immunoblotted using Mab N9 as described below.

Preparation of Degranulated Plasma Membranes

Freshly isolated human neutrophils were DFP treated, stimulated with fMLF in the presence of 1 μM fMLF, N_2 cavitated, and the post nuclear supernatant obtained as described above. Post nuclear supernatants were then sedimented at 100,000 xg for 30 min (4°C) in a Ti-60 rotor to isolate a membrane fraction. Importantly, neutrophils degranulated under the conditions of fMLF stimulation imposed in this method are largely devoid of internal granules (Mukherjee et al., 1994) so that the cellular membranes isolated from post nuclear supernatants are predominantly of plasma membrane origin. Membrane pellets were accordingly resuspended by light sonication on ice and stored at -70°C in 1-5 ml aliquots ($5x10^8$ cell equivalents/ml) until use.

Membrane Washes

Frozen aliquots of degranulated membranes were thawed in the presence of 100 μM PMSF and protease inhibitor cocktail (P8340), lightly sonicated and sedimented at 100,000 xg for 30 min (4°C) in a TLA 100.2 rotor, and resuspended in either a 1 M NaCl solution or a 10 mM NaOH solution followed by sedimentation as above. Control runs were conducted in parallel in which membranes were resuspended in DPBS, pH 7.4. Membrane pellets were finally resuspended in DPBS, separated by SDS-PAGE, and immunoblotted as described below.

Triton X-114 Phase Separation

This method was performed as described by Borregaard and Tauber (Borregaard and Tauber, 1984). Briefly, 50 ml of a 2% Triton X-114 stock solution was prepared in 15 mM glycine and 3 mM Tris-HCl, pH 8.6 and maintained on ice. The Triton X-114 solution was then incubated at 37°C until cloudy and immediately sedimented at 1500 xg for 20 min (37°C). The supernatant was discarded and the Triton X-114 pellet resuspended in the above buffer (4°C) and the process repeated atotal of three times. The final Triton X-114 pellet represents a final working stock of 11.4% detergent used in the assay. For the assay, the 11.4% stock solution was diluted to 0.7% in cold 150 mM NaCl and 10 mM Tris-HCl, pH 7.4. One milliliter of membrane suspension (~2.5 mg protein) was solubilized in 4ml 0.7% solution for 30 min at 4°C. Insoluble material was removed by sedimentation at 100,000 xg for 30 min (4°C) and the supernatant heated at 37°C until cloudy in appearance and then sedimented at 1500 xg for 20 min (37°C). The resulting pellet fraction, representing the TX-114 phase, was diluted to its original 5 ml volume in

150 mM NaCl and 10 mM Tris-HCl, pH 7.4, and it and the supernatant were examined for relative hCap content by immunoblot analysis, conducted as described below.

In-Gel Tryptic Digestion

Following SDS-PAGE, gels were stained with Labsafe Gel Blue (G Biosciences) according to the manufacturer's instructions. For tryptic digestion, gel spots were excised, placed in microcentrifuge tubes and washed for 3 x 10 min in 200 µl 25 mM NH_4HCO_3 (pH-8.0), 50 % acetonitrile. Washed gel slices were then dried in a vacuum centrifuge and rehydrated in 75 µl of 25 mM NH_4HCO_3 (pH-8.0), 10 mM DTT for 30 min at 56 °C. Reduced gel slices were then dehydrated with 200 µl of acetonitrile and incubated with 100 µl of 25 mM NH_4HCO_3 (pH-8.0), 55 mM iodoacetamide in the dark for 20 min at room temperature. The gel slices were then washed for 10 min at room temperature with 200 µl of 25 mM NH_4HCO_3 (pH-8.0) followed by dehydration in 200 µl of acetonitrile. After washing gel slices three times in the above fashion, they were dried in a vacuum centrifuge and rehydrated with 50 µl of 25 mM NH_4HCO_3 (pH-8.0), 10 % acetonitrile, 40 ng/µl trypsin on ice for 30 min. Excess digestion buffer was then removed from the rehydrated slices, 20-40 µl of 25 mM NH_4HCO_3 (pH-8.0), 10 % acetonitrile was added and digests were incubated overnight at 37 °C. Following overnight digestion, the gel slices were placed in a bath sonicator for 10 minutes and the digest solution was removed from the gel slices (aqueous extract). The gel slices were then extracted with 30 µl of 50% actetonitrile, 0.1% trifluoroacetic (TFA) acid by bath sonication for 10 minutes (organic extract). The aqueous and organic extracts were then

pooled, dried in a vacuum centrifuge and resuspended in 15 μl of 50% acetonitrile, 0.1% TFA for MALDI analysis.

Immunoprecipitation Assays

Monoclonal antibodies H7, N9, and K16 were separately conjugated to CNBr activated sepharose beads according to manufacturer's directions. Labeled beads were incubated with purified cellular membranes or purified plasma membrane fractions as indicated in the text from ~$5x10^7$ – $1x10^8$ cell equivalents in a final volume of 1.4 ml (1:1 vol ratio) in PBS, pH 7.4, with or without 1% DDM as indicated in the text. Incubations were for 1hr at 25°C. Beads were then washed 6-8 times in PBS, pH 7.4, and 1% BSA with or without 0.2% DDM, denatured in βME-SDS for 5 minutes (90°C), and separated by SDS-PAGE as indicated below.

MALDI Mass Spectrometry

For MALDI analysis, fractions were removed from the digests and diluted 2-fold with a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile:0.1% TFA, with 1 μl of this resulting mixture spotted on the MALDI plate and allowed to air dry. MALDI analysis was conducted on a Bruker BiFlexIII mass spectrometer in reflectron mode with positive ion spectra averaged over 100-600 laser shots between a mass range of 500-5000 Da. The instrument was externally calibrated using the singly protonated monoisotopic masses of either bradykinin fragment 1-7 (757.39), angiotensin II (1046.53 Da), and adrenocorticotropin hormone 18-36 (2465.19 Da). For protein identification, mass spectral data was entered into the program Mascot

(http://www.matrixscience.com/search_form_select.html) where the human protein database was searched allowing for the variable modification of both Cys (carbamidomethyl) and Met (oxidation) residues.

Alkaline Phosphatase Activity

Alkaline phosphatase activity was measured in individual gradient fractions by synchronous addition of 8 mM p-nitrophenylphosphate substrate (DeChatelet and Cooper, 1970) in 0.1 M diethanolamine-HCl, pH 9.75, and 0.33 mM $MgCl_2$ followed by 22 minute incubation (37°C). Reactions were terminated by addition of 100 mM NaOH (final concentration) and signal quantitated at wavelength 405nm using a microtiter plate reader.

SDS-PAGE/Immunoblot Analysis

SDS-PAGE. Gradient fractions from isopycnic sucrose density sedimentation or material from immunoprecipitation assays were denatured in βME-SDS for 5 minutes (90°C), loaded onto 9-15% gradient gels, separated by SDS-PAGE, and either stained for protein (see above) or immediately transferred onto PVDF membranes for immunoblot analysis. In some instances, gels stained for protein were de-stained, electrotransferred, and immunoblotted as described below.

Immunoblot Analysis. PVDF membranes were blocked in 0.5 M NaCl, 10 mM HEPES, pH 7.4, 3% BSA and 10% goat serum overnight (4°C). Primary antibody (800 ng N9 or H7; 3 μg rabbit anti-human lactoferrin) was applied to coated membranes in

DPBS, pH 7.4, 0.2% Tween 20, 1% BSA and 3% goat serum for 1 hr (25°C). Membranes were rinsed 5 times for 25 minutes total (25°C) in 0.25 M NaCl, 10 mM HEPES, pH 7.4, 0.2% Tween 20. A 1:20,000 dilution of secondary anti-IgG antibody was applied as described above and membranes rinsed as above. Signal was visualized using Supersignal chemiluminescence kit and quantitated with ImagQuant program software, version 3.3.

Structural Modeling

The predicted secondary structure of hCap was obtained using 3D-PSSM software (Kelley et al., 2000), available free on the internet from the Biomolecular Modeling Laboratory of the Imperial Cancer Research Fund (London, U.K) website at www.bmm.icnet.uk/~3dpssm. This program first selects proteins similar to the query sequence from a protein library of known 3-D structures. Proteins identified from this first round of selection are then used to build a database of known 3-D structures similar to the unknown sequence. Proteins that score highest in terms of 1-D sequence alignment, matching of predicted secondary structural elements, and solvent accessibility are then used by a "threading" algorithm to determine the predicted secondary structure of the protein of interest. Based on these criteria, the protein with the lowest e-value, and thus highest degree of structural similarity, to hCap was the porcine cathelicidin, protegrin-3. Structural predictions are shown in tabular form (Fig 12) for the first 50 amino acids of the pro/cathelin domain.

Results

Mabs H7 and N9 were produced, together with a number flavocytochrome b-specific Mabs, from normal BALB/c mice using detergent-solubilized, partially purified preparations of human neutrophil integral membrane protein as immunogen (Burritt et al., 1995). Hybridoma cells testing positive against crude preparations of cytochrome b in subsequent ELISA assays and/or immunoblots were further cloned by limiting dilution. hCap was later identified as a contaminant of these partially purified cytochrome b

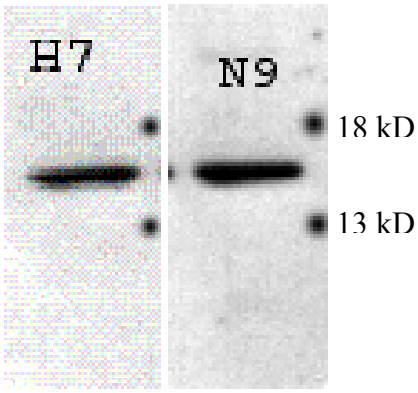


Figure 1. Monoclonal antibodies N9 and H7 recognize a single ~16 kD band. Cellular membranes prepared from freshly isolated neutrophils processed by SDS-PAGE and electro-transferred onto PVDF for immunoblot analysis with N9 or H7 monoclonal antibodies.

preparations (A. V. Jesaitis, J. B. Burritt, A. J. Jesaitis, data not shown), due to its capacity to associate surface membranes (see below). As shown in Figure 1, Mabs N9 and H7 both specifically recognized a single band at ~16 kD present in cellular membrane preparations from purified human neutrophils. The similarity of blotting

patterns under a variety of conditions in different samples (not shown) suggested that both Mabs may be targeting the same protein.

N-Terminal Sequence Analysis

The identity of the antigen bound Mab H7 was initially determined by N-terminal sequence analysis. Protein was purified by immunoprecipitation and processed for sequence analysis by SDS-PAGE and electroblotting onto PVDF membranes. Table 1 compares the alignment of the first eight N-terminal residues of the antigen identified by

Table 1: N-terminal sequence analysis of H7-bound antigen

Protein	N-Terminal Sequence
Antigen	SSDANLYR
hCap	SSDANLYR

Comparison of the first eight amino acids of the 16 kD antigen recognized by Mab H7, obtained by N-terminal sequence analysis, with corresponding residues in hCap primary structure.

sequence analysis with the corresponding residues of hCap primary structure. The identical correspondence of residues between the two proteins provided initial evidence that the 16 kD antigen recognized by Mabs N9 and H7 is hCap.

MALDI Analysis of Immunopurified Antigen

Following its secretion by cells, the native form of hCap, estimated at ~18 kD, is cleaved by specific proteases into peptide products estimated at 14 kD and 4 kD. As the relative molecular weight of the antigen detected by Mabs H7 and N9 was between 14,000-18,000, MALDI mass spectrometry was used to determine the molecular form of hCap represented by the 16 kD antigen. The SDS-PAGE profile of proteins present in the eluates of H7 and the irrelevant Mab (K16) revealed several distinct bands, including a 16 kD band present only in H7 eluate and a 14 kD band found in the bound material of

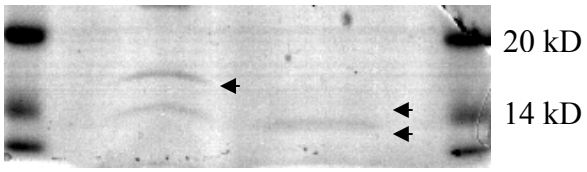


Figure 2. Immunoprecipitation of neutrophil cellular membranes using Mabs H7 and K16. Cellular membranes (including plasma membranes and internal organelles) were isolated from N2 cavitated (unstimulated) neutrophils by high speed sedimentation, solubilized in 1% DDM, and incubated with CNBr activated beads conjugated to either H7 or N9. Eluates collected from each antibody were separated by SDS-PAGE and stained for protein content as described in Materials and Methods. Single and double arrowheads indicate16 kD and 14 kD bands, respectively.

of both Mabs (Figure 2). The 16 kD and 14 kD bands were trypsin-digested, and the peptide mass profiles obtained from MALDI analysis were evaluated against both the human protein data base and against theoretical digests of hCap and lysozyme, respectively, using web-based programs available from Matrix Science and ExPASy proteomic servers, respectively. Significant probability scores were obtained for hCap (16 kD) and lysozyme (14 kD), with respective coverages of 55% (pro/cathelin domain) and

Table 2. Identification of hCap by MALDI mass spectrometry of trypsin-digested H7 eluate from immunoprecipitations using purified detergent-solubilized membranes.

Observed Mass (Da)	Theoretical Mass (Da)	Error (Da)	Identified Residues
515.243	515.3300	-0.09	153-156
531.22	531.3137	-0.09	102-106
550.224	550.2983	-0.07	159-162
579.263	579.3249	-0.06	149-152
587.279	587.3511	-0.07	37-41
598.303	598.3671	-0.06	163-167
671.348	671.4311	-0.08	152-156
791.479	791.4774	0.00	157-162
886.442	886.4741	-0.03	42-49
925.431	925.4374	-0.01	50-57
1085.539	1085.5778	-0.04	132-140
1193.495*	1193.5765	-0.08	108-118
1213.558	1213.6728	-0.11	132-141

*Contains (1) alkylated cysteine and (1) methionine sulfoxide residues

Table 3. Identification of lysozyme by MALDI mass spectrometry of trypsin-digested H7 eluate from immunoprecipiations using purified detergent-solubilized membranes.

Observed Mass (Da)	Theoretical Mass (Da)	Error (Da)	Identified Residues
529.368	529.3569	0.01	98-101
550.159	550.2983	-0.14	2-5
678.411	678.3933	0.02	1-5
685.377	685.3627	0.01	102-107
788.458	788.4202	0.04	108-113
811.407*	811.3767	0.03	15-21
933.487**	933.5186	-0.03	6-13
967.474*	967.4778	0.00	14-21
981.445	981.4384	0.01	42-50
1012.433	1012.4483	-0.02	34-41
1058.515	1058.5642	-0.05	108-115
1400.517	1400.6804	-0.16	51-62

*Contains methionine sulfoxide residue. **Contains alkylated cysteine residue.

Table 4. Identification of lysozyme by MALDI mass spectrometry from trypsin-digested K16 eluate from immunoprecipitations using purified detergent-solubilized membranes.

Observed Mass (Da)	Theoretical Mass (Da)	Error (Da)	Identified Residues
529.337	529.3569	-0.02	98-101
550.143	550.2983	-0.15	2-5
678.391	678.3933	0.00	1-5
685.356	685.3627	0.00	102-107
788.408	788.4202	-0.01	108-113
981.401	981.4384	-0.03	42-50
1012.365	1012.4483	-0.08	34-41
1400.491	1400.6804	-0.18	51-62

65% (cationic peptide) for hCap, and 43% for human lysozyme (Tables 2-3, 8). The 14 kD band present in immunoprecipitates from irrelevant (K16) Mab also yielded a significant probability score for lysozyme with 31% coverage (Tables 4, 8). These data indicate that the 16 kD antigen specifically recognized by Mabs H7 and N9 is native form of hCap.

A

N9
REEPD**ARGY**
SSVV**N**Q**RGY**
WLG**PNSRG**P
WLG**PNSRGY**
GIVV**NARGY**
GDLP**NTRGY**

NARGY→ NARGZ = Consensus

B

1 mktqrdghsl grwslvllll glvmplaiia qvlsykeavl raidginqrs sdanlyrlld 60

61 ldprptmdgd pdtpkpvsft vketvcprtt qqspedcdfk kdglvkrcmg 110

Pro/cathelin Active Peptide

111 tvtl **n**q**arg**s **f**disc dkdnk rfallgdffr kskekigkef krivqrikdf lrnlvprtes 170

C

n arg z
111 tvtl**n**q**arg**s**f**discdkdnk 130

Figure 3. Epitope mapping of N9 by phage display analysis. A) sequences from phage clones selected against N9 (left) and H7 (right) with common residues in bold type, were

(Figure 3 –continued) used to obtain the five amino acid consensus motif, NARGZ, where 'Z' indicates an aromatic amino acid. B) Primary sequence of the native form of hCap. Dotted and solid lines indicate the N-terminal pro/cathelin and active peptide regions. Hydrophobic residues present within the N-terminal region of pro/cathelin domain are underlined. The N9/H7 epitope identified by the phage consensus sequence in (A). Amino acids corresponding to consensus residues include residues 115, 117-19 and 121, shown in bold type and located near C-terminus of the pro/cathelin domain, within the region linking the pro/cathelin-domain and the active, C-terminal cationic peptide. **C**) Alignment of the consensus sequence shown in (A) with corresponding amino acids of the C-terminal region of the pro/cathelin domain, which are underlined in bold. The general correspondence between phenylalanine (121) and tyrosine, within hCap primary structural and phage consensus motifs, respectively, is represented by inclusion of Z in place of Y in the NARGY consensus, as indicated in (A). The consensus alignment is made partially discontinuous by the presence of two amino acids, glutamine (116) and serine (120), in primary structure that are absent in the consensus sequence.

Phage Display Mapping of Mabs N9 and H7

The composition and structure of the amino acid sequence motif targeted by Mabs N9 and H7 was next examined using phage display analysis. Phage clones bearing peptides mimicking the epitope of each Mab were accordingly isolated and sequenced (Burritt et al., 1996).

Sequence analysis revealed a remarkable similarity in the phage displayed peptides bound by N9 and H7, such that virtually identical consensus motifs were obtained for each case (Figure 3A), suggesting that each Mab recognizes a similar or identical epitope sequence in hCap primary structure. An initial consensus sequence, NARGY, was obtained from phage clones specific for the antigen binding domains of N9 or H7 Mabs, and this consensus was later modified to NARGZ, where 'Z' is an aromatic residue. The NARGZ consensus has been assigned to residues 115, 117-119 and 121 located at the C-terminal end of the pro-sequence (Figure 3C), which comprises the first 132 amino acids of hCap primary structure (Figure 3B) and referred to in present study as

the pro/cathelin domain. The primary function of the pro/cathelin domain (dotted line, Figure 3B) is to sequester and inactivate the antimicrobial function of the 38-residue cationic antimicrobial C-terminal region (solid line, Figure 3B).

In Vitro Physiologic Cleavage of hCap in Isolated Neutrophils

As the functional activation of native hCap results in enzymatic release of the ~4 kD C-terminal peptide from the ~14 kD pro/cathelin domain, the location of the consensus motif in primary structure suggests that Mabs N9 and H7 recognize both the native form of hCap and the free ~14 kD N-terminal pro/cathelin domain following protease cleavage (Figure 3B). To test this possibility, freshly isolated human neutrophils were therefore stimulated with fMLF in the presence of cytochalasin B to obtain fully activated, degranulated cells (Mukherjee et al., 1994). One consequence of simultaneous azurophilic (primary) and specific granule exocytosis resulting from neutrophil activation in the presence of cytochalasin B is the exposure of specific granule hCap to proteases present in primary granules that catalyze hCap cleavage, thus releasing the C-terminal antibiotic peptide from the N-terminal pro/cathelin domain. This cleavage, predominantly mediated by serine proteinases, has been shown to occur in response to incubation conditions similar to those used in these studies (Sorensen et al., 2001). Therefore supernatants from degranulated neutrophils were collected and analyzed by immunoblot analysis for both the native and cleaved forms of hCap. Figure 4 shows that two bands are recognized from immunoblots made with supernatants obtained from degranulated neutrophils at relative molecular weights of ~16 kD and ~14 kD. The latter band falls within the molecular weight range (12-14 kD) expected for the pro/cathelin domain

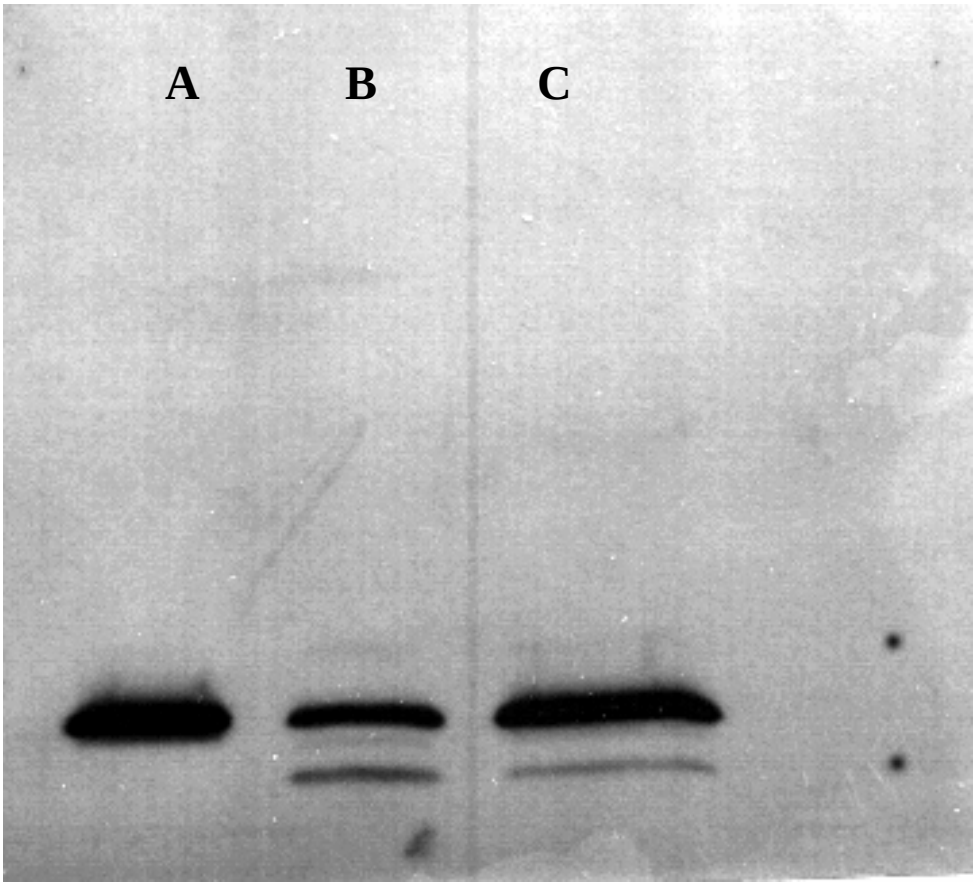


Figure 4. Specific binding of Mab N9 to16 kD and14 kD proteins present in supernatant from degranulated neutrophils. Neutrophils were stimulated with fMLF in the presence of cytochalasin B as described in Materials and Methods. After terminating cellular activation, supernatants were isolated, concentrated by ultrafiltration, and analyzed by immunoblot with Mab N9 or H7 (data not shown; each antibody gave indistinguishable blotting patterns). Lane A, eluate from H7-precipitation of antigen from detergent-solubilized degranulated membranes. Lane B, supernatant from degranulated neutrophils. Lane C, 50:50 mixture of eluate from lane A and supernatant from lane B.

(Sorensen et al., 2003), and identification of this fragment as the N-terminal pro/cathelin fragment of hCap was supported by MALDI analysis.

MALDI Analysis of the Putative 14 kD Proteolytic Product of hCap

To confirm the identity of the ~14 kD band recovered from the supernatant of fully degranulated neutrophils by immunoblot analysis as a proteolytic fragment of the native 16 kD hCap (Figure 4), the immunopurified protein was digested with trypsin and analyzed by MALDI mass spectrometry as indicated above. Both the 16 kD and 14 kD bands were recovered from H7 eluate, as indicated in the protein gel shown in

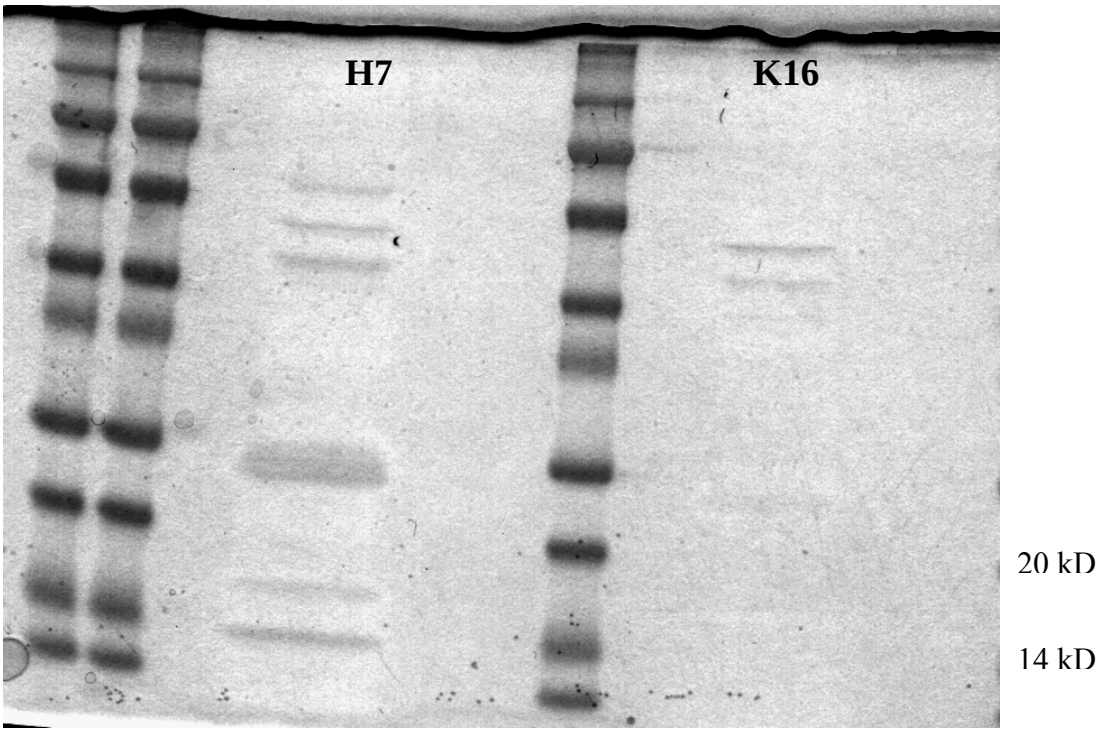


Figure 5. Protein recovered from H7 and K16 eluates following imunoprecipitation of degranulated neutrophil supernatants. Eluates from H7 (left) and K16 (right) were solubilized and denatured in SDS sample buffer, separated on a 9-15% polyacrylamide gradient gel, and stained from protein content as described in Material and Methods. The

(Figure 5 –continued) 16 kD and 14 kD bands present in the H7 eluate (indicated by the arrowheads) were removed from the gel and further processed for MALDI analysis.

Figure 5, and processed for mass spectrometry. Interestingly, the protein stain from the K16 (control) Mab did not reveal the nonspecific presence of lysozyme (14 kD) observed in immunoprecipitation assays using detergent-solubilized membranes (Figure 2). By contrast, lysozyme was identified as a component of the 14 kD band from H7 eluate (see below). When the profile of peptide masses obtained from the 16 kD antigen was analyzed, a single protein species, the native hCap, was identified with 50% sequence coverage (Tables 5, 8), consistent with spectra obtained from membrane-isolated 16 kD antigen (Table 2). Results obtained from the mass analysis of the trypsin-digested 14 kD band yielded more complex results due to the nonspecific contamination of lysozyme initially identified in immunoprecipitation assays employing detergent solubilized membranes (Figs 2, 10a; Tables 3, 8). Accordingly, the mass analysis obtained from the 14 kD band of the H7 eluate suggested the presence of both lysozyme (Table 7) and the pro/cathelin domain (Table 6) with respective sequence coverages of 49% and 25% (Table 8). The lysozyme contaminant found in the 14 kD band was expected because of the nonspecific binding of Mab H7 to this protein (see above) and evidence that lysozyme is a highly charged, relatively abundant, soluble granule protein that is released into the extracellular milieu during neutrophil degranulation (Ambruso et al., 1984;Mutasa, 1989). These data suggest that lysozyme is capable of adhering to neutrophil surface membranes in a regulated manner with relatively high affinity. In addition, other peptide masses present in the 14 kD band mapped to sequences in the N-terminus of hCap (Table 6), thus verifying the presence of the pro/cathelin domain of hCap first detected by

Table 5. Identification of hCap by MALDI mass spectrometry of trypsin-digested H7 eluate from immunoprecipitations using degranulated cell supernatant.

Observed Mass (Da)	Theoretical Mass (Da)	Error (Da)	Identified Residues
598.429	598.3671	0.06	163-167
886.518	886.4741	0.04	42-49
925.482	925.4374	0.04	50-57
1085.6	1085.5778	0.02	132-140

Table 6. Identification of hCap pro/cathelin domain by MALDI mass spectrometry from trypsin-digested H7 eluate from immunoprecipitations using degranulated cell supernatant

Observed Mass (Da)	Theoretical Mass (Da)	Error (Da)	Identified Residues
515.184	515.3300	-0.14	153-156
550.023	550.2983	-0.27	159-162
886.518	886.4741	0.04	42-49
925.482	925.4374	0.04	50-57

Table 7. Identification of lysozyme by MALDI mass spectrometry from trypsin-digested H7 eluate from immunoprecipitations using degranulated cell supernatant

Observed Mass (Da)	Theoretical Mass (Da)	Error (Da)	Identified Residues
788.336	788.4202	-0.08	108-113
811.295	811.3767	-0.08	15-21
981.361	981.4384	-0.07	42-50
1012.370	1012.4483	-0.07	34-41
1400.494	1400.6804	-0.18	51-62

Table 8. Coverage and probability scores of antigens isolated

IP Bait	Species Recovered	% Coverage hCap: pr/pe	% Coverage Lysozyme	Probability Score
H7 (s.m.)	16 kD 14 kD	55/65 --	-- 43	18* 75*
K16 (s.m.)	14 kD	--	31	93*
H7 (s.d.)	16 kD 14 kD 14 kD	50 25 --	-- -- 49	82* 67.5* 89*

Peptide fragmentation patterns were analyzed using the Mascot program available on the internet at matrixscience.com. *Significance thresholds were 76 and 64 for hCap (s.m.) and lysozyme (s.m.), respectively, and 64 for the native (s.d.; 16 kD), pro/cathelicidin (s.d.; 14 kD) forms of hCap and lysozyme (s.d.). IP = immunoprecipitation; pr/pe = (native) protein/(cationic) peptide; s.m. = IP using solubilized membranes; s.d. IP using supernatants from fMLF-stimulated degranulated neutrophils.

immunoblot analysis (Figure 4), and consistent with previous studies identifying the 14 kD pro/cathelin domain as the primary cleavage product of native hCap (Sorensen et al., 2001). The presence of the hCap 14 kD pro/cathelin domain could not be detected in H7/N9 eluates from immunoprecipitation assays, as would be expected with a regulated cleavage of hCap that occurs in association with primary granule loss (Figure 2; Tables 3, 8). Rather, these experiments employed detergent-solubilized membrane fractions prepared from the post-nuclear fractions of unstimulated neutrophil cavitates. Collectively, these data are consistent with identification of immuno-purified antigen as hCap by N-terminal sequence analysis (Table 1) and MALDI mass spectrometry (Tables 2, 8), as well as with consensus sequence data obtained from phage epitope mapping Mabs N9 and H7 indicating specific recognition of the N-terminal pro/cathelicidin domain of hCap (Figures 3 B-C).

Subcellular Fractionation Pattern of
Antigen in Resting and fMLF-Stimulated Neutrophils

As hCap expressed by human neutrophils is selectively enriched in specific (secondary) granules, additional experiments were undertaken to confirm its subcellular distribution in resting (non-activated) neutrophils. Post-nuclear material from freshly isolated, N_2 cavitated neutrophil populations were separated by isopycnic density gradient sedimentation, and processed by SDS-PAGE and subsequent immunoblot analysis with N9 (n = 3) and H7 (n = 3; data not shown). For these experiments, lactoferrin (LF) was used as a marker protein for specific granules, and alkaline phosphatase (AP) was used to identify gradient regions of plasma membrane

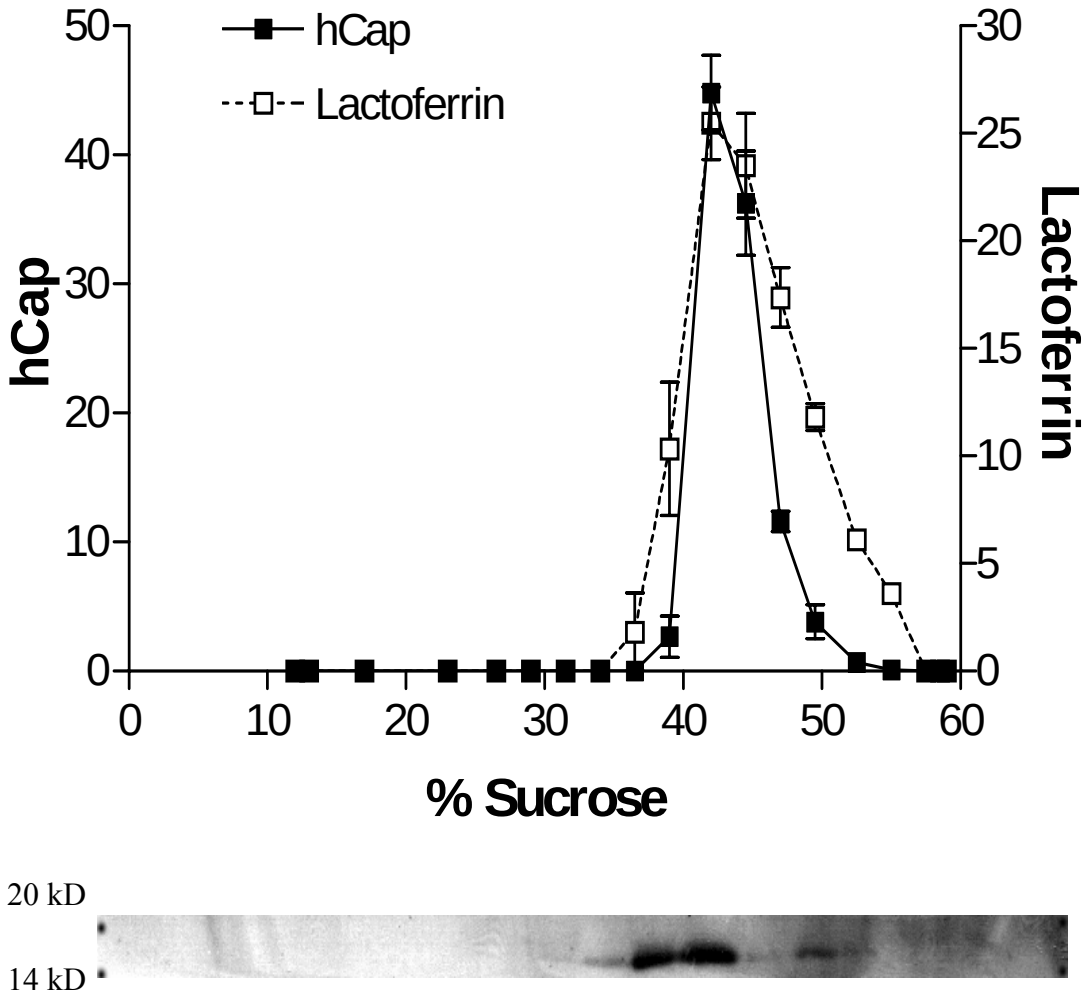


Figure 6. The 16 kD antigen recognized by N9 is enriched in specific granules of human neutrophils. Freshly isolated, nonactivated neutrophils were nitrogen-cavitated and post-nuclear fractions from low speed sedimentation of cavitates fractionated by isopycnic sucrose density sedimentation. Equal aliquots of gradient fractions collected were processed for immuno-blotting with N9 or the specific granule marker, lactoferrin as described in Materials and Methods. Data are depicted as % total activity as a function of % sucrose. A representative film depicting the immuno-staining pattern of N9 is included below the graph. Data shown are averaged from three independent experiments.

enrichment (see Figure 7). As shown in Figure 6, plasma membrane-enriched vesicles have a peak distribution at ~30% sucrose that is clearly resolved from the peak density sedimentation of intact specific granules at ~42% sucrose. The immunoblot in Figure 6 shows detection of a single band at a molecular weight of ~16 kD that is characteristic of the native (uncleaved) form of hCap (Tables 2 and 8). The blotting pattern of N9 suggests co-sedimentation with lactoferrin, consistent with specific granule localization as well as the identity of the ~16 kD antigen as the native form of hCap. The results obtained with H7 were indistinguishable from the results shown for N9.

As the nature of the immunogen used generate hybridomas producing the N9 and H7 Mabs suggested an association of N9/H7 antigen (hCap) with the surface membranes of activated neutrophils, the subcellular localization pattern of hCap was further assessed using activated neutrophil populations. Post-nuclear fractions from N_2 cavitated, fMLF-stimulated neutrophil populations were N_2 cavitated followed by density gradient fractionation separated by isopycnic sucrose density sedimentation. Fractions were then analyzed by immunoblot and enzymatic assay. The marker proteins used to identify the subcellular distribution of plasma membrane (AP) and specific granule (LF) fractions are the same as those used in Figure 6. We found that stimulation of isolated neutrophils with 1 μM fMLF led to a 37-60% depletion of intact specific granules in the populations examined (n = 4; Figure 7). The antigen remaining within intact specific granules gave a sufficiently strong signal to permit direct comparison of plasma membrane-bound and specific granule-localized hCap after immuno-staining with either N9 (n = 3) or H7 (n = 3; data not shown).

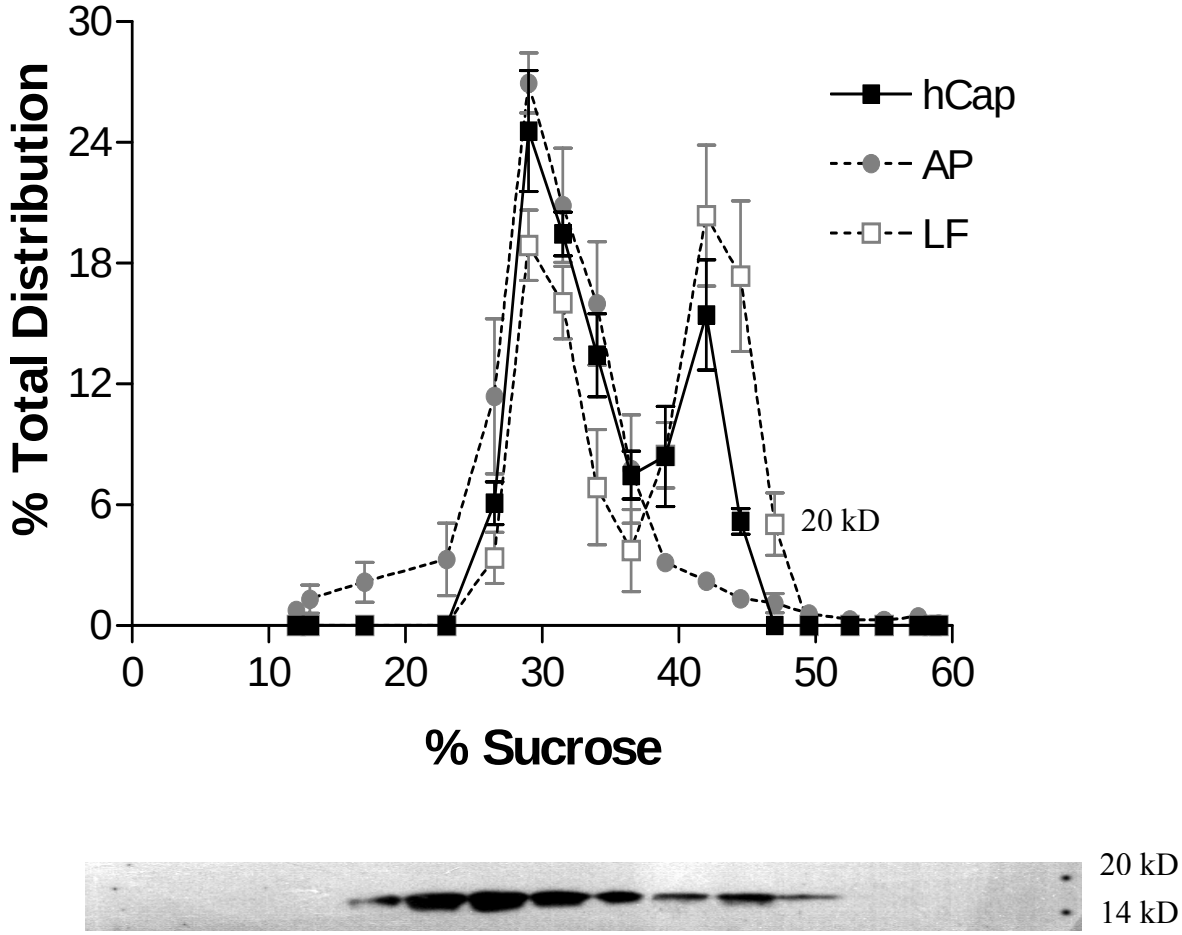


Figure 7. The cell surface-localized antigen recognized by N9 corresponds to the native hCap molecule enriched within intact specific granules. Gradient fractions from isopycnic sucrose density gradient sedimentation of N_2 cavitates prepared from fMLF-stimulated neutrophils were either enzymatically assayed for alkaline phosphatase (AP) activity or processed by SDS-PAGE, electro-transferred and immuno-blotted with N9 or lactoferrin (LF) as described in Materials and Methods. Data are depicted as % total activity as a function of % sucrose. A representative film depicting the immuno-staining pattern of N9 is included below the graph.

Immunoblots performed with N9 detected a single band in plasma membrane fraction of ~16 kD, which corresponded in relative molecular weight to the native form of hCap present within intact specific granules (n = 3; Figure 7). The results obtained with H7 were indistinguishable from the results shown for N9. The data shown in Figure 7 additionally suggests co-sedimentation of LF with surface membrane AP activity, consistent with previous reports demonstrating the high affinity association of lactoferrin with activated neutrophils (Oseas et al., 1981;Boxer et al., 1982). Indeed, the subcellular localization patterns of LF differ between resting and fMLF-activated neutrophil populations in a manner analogous to that of hCap (compare Figures 6 and 7). The high affinity, functional association of LF with activated neutrophil plasma membranes has been described in detail in several previous studies (Afeltra et al., 1997;Manev et al., 1998). Such studies suggested the selective expression of LF binding sites on surface membranes of activated human neutrophil populations and are consistent with the patterns of LF sedimentation shown in Figures 6 and 7. This conditional bimodal subcellular distribution of LF is paralleled by hCap and may indicate that hCap surface expression is similarly regulated. Taken together, these data suggest a newfound capacity of the native (uncleaved) hCap molecule to associate with surface membranes of activated neutrophils.

Immunoprecipitation of hCap from fMLF-Stimulated Neutrophil Plasma Membranes

Immunoblot analysis of various preparations of plasma membranes prepared from

partially degranulated fMLF-stimulated neutrophil populations [Figure 7, (Parkos et al., 1987)] indicated that the membrane associated antigen had a relative molecular weight of 16 kD. To test whether the selective distribution of hCap within gradient regions of plasma membrane enrichment accurately reflected cell surface-localized (as opposed to internalized) antigen in fMLF-activated neutrophils, we sought to immunopurify hCap present from the cell-surface of this population using N9-conjugated sepharose beads and beads conjugated with an irrelevant antibody (K16). Mab-conjugated beads were separately incubated with purified plasma membranes obtained by the differential sedimentation of post-nuclear fractions from N_2 cavitated, fMLF-stimulated neutrophils. Because incubations with immunobeads were performed in the absence of detergent, this approach led to the isolation of membrane fragments co-expressing the N9 antigen and other assorted membrane-associated proteins. Use of detergent was avoided during immunoprecipitation to insure Mab binding to only surface exposed hCap, in case of minor contamination of membrane preparations with intact specific granules. Following one-hour incubation at 25°C, beads were thoroughly washed in PBS with 1% BSA and the bead-bound material processed for immunoblotting. Immunoblots performed with material from N9 immunoprecipitations yielded identical immunoblot results when probed with N9 or H7. The immunoblot shown at the top of Figure 8 was probed with N9 and indicates that the 16 kD native hCap is the only form of the molecule present on the cell surface. Indistinguishable data were obtained when immunoblots were stained with H7. These results are consistent with the data shown in Figure 7 directly comparing

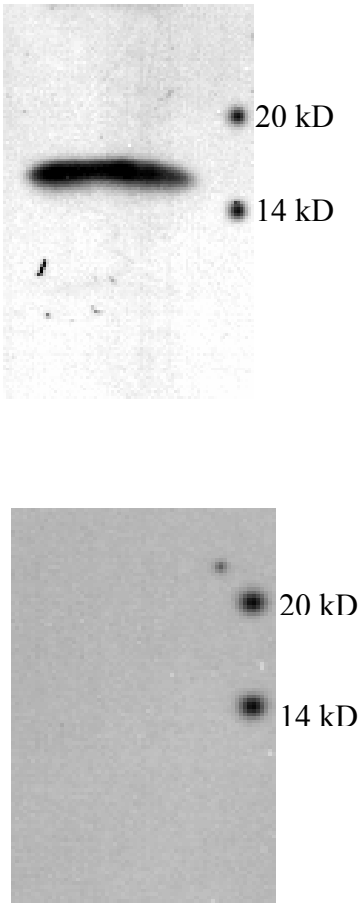


Figure 8. The cell surface antigen recognized by Mab N9 corresponds to a single ~16 kD band on fMLF-stimulated neutrophils. Plasma membranes were isolated from freshly prepared neutrophil populations as described in Materials and Methods and exposed to N9-conjugated sepharose beads, or to beads identically prepared using control monoclonal antibody K16, in the absence of detergent extraction. Beads with bound material were processed for immunoblot analysis with N9 as described in Materials and Methods. The top and bottom films are representative of three independent experiments and show N9 activity against bead-bound material from N9 and K16 immunoprecipitations, respectively.

the surface-bound hCap with the native, uncleaved form housed in intact specific granules. No activity was detected when N9 (or H7) was applied to immunoblots from K16-bound material (n = 3; Figure 8, bottom).

Extent of hCap-Plasma Membrane-Association is Regulated by Progressive Changes in the Neutrophil Activation State

The data presented above suggested the differential localization of the native hCap molecule on the surface of resting versus activated neutrophil plasma membranes. As noted above, specific granule LF likewise adheres to the cell surfaces of activated neutrophils (Figure 7; (Swain et al., 2000)) in a functionally significant interaction (Manev et al., 1998). Because the functional activation of native hCap is similarly linked to dynamic changes in the neutrophil activation-state, we investigated the capacity of hCap to bind the cell surface of intact neutrophils during distinct stages of cellular activation. When resting (nonactivated), fMLF-stimulated, and fully degranulated neutrophil populations were examined for cell-surface antigen, a positive correlation was found between the extent of surface localization and progressive stages of neutrophil activation, with hCap surface-labeling varying as much as ~40-fold when resting and fully degranulated cellular states were compared (n = 3; Figure 9). Both N9 and H7 gave equivalent patterns of detection in both molecular size and subcellular localization. Only data from N9 are shown in Figure 9.

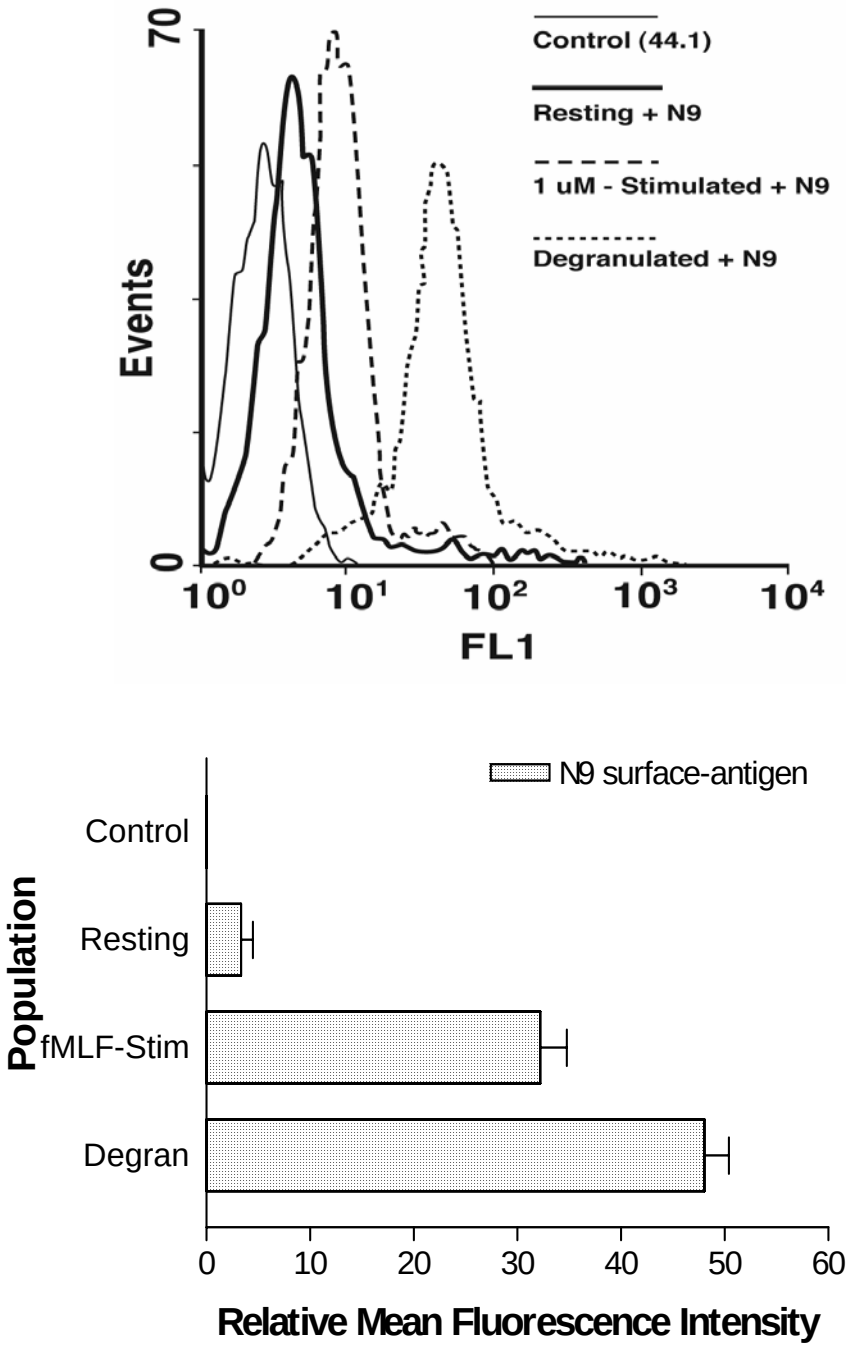


Figure 9. N9 recognition of intact neutrophil populations is regulated by changes in cellular activation-state. Freshly isolated neutrophils were prepared as described in Materials and Methods, immuno-labeled with N9 and analyzed by FACs. A sample

(Figure 9 –continued) histogram is shown above with bar graph below. Data from the bar graph are expressed as relative mean fluorescence intensity of surface antigen expression and are pooled from three independent experiments. The negative controls employed yielded similar results and included either 2º antibody alone, irrelevant (K16) monoclonal, or a monoclonal antibody (44.1) specific for the cytoplasmic domain of the p22 component of the cytochrome b heterodimer. Only data from the 44.1 control is shown.

Immunoprecipitation of hCap from the
Plasma Membranes of Fully Degranulated Neutrophil Populations

Flow cytometry of fMLF-stimulated and degranulated neutrophils suggested localization of the N9/H7 antigen to the cell surface of such stimulated neutrophils. The immunoblotting patterns from supernatants obtained from degranulated neutrophils indicated the presence of two molecular weight forms of antigen recognized by Mabs H7 (Figure 5) and N9 (data not shown). As this data together with the observed increase in hCap association with degranulated plasma membrane membranes, relative to partially degranulated, fMLF-stimulated populations, (Figure 9) suggested that both the native 16 kD form and protease-cleaved 14 kD prodomain could be contributing to the enhanced staining pattern of this population, the relative molecular weight of antigen associated with degranulated neutrophil plasma membranes was further examined. Neutrophil populations were degranulated by stimulation with fMLF in the presence of cytochalasin B and then subjected to N_2 cavitation followed by isolation of the plasma membrane fraction from post-nuclear supernatants (Mukherjee et al., 1994). Membrane fractions were washed in 1M NaCl to remove loosely adherent hCap (see below) and solubilized in detergent. Prior to immunoprecipitation, membrane extracts were sedimented at 100,000 x g to remove insoluble material.

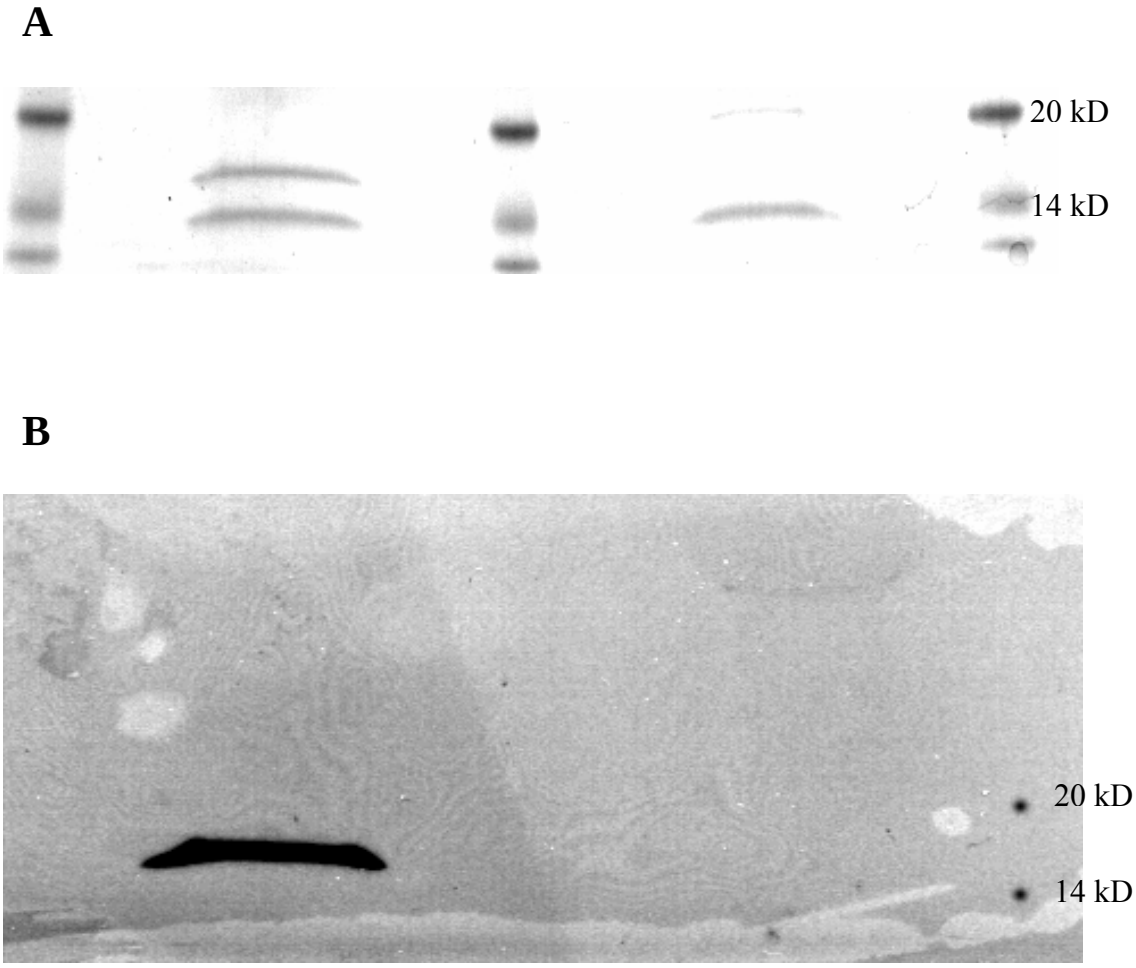


Figure 10. Immunoprecipitation of 16 kD antigen from degranulated plasma membranes. Degranulated neutrophil plasma membranes were isolated as described in Materials and Methods, solubilized in n-dodecyl ß-D-maltoside, and immunoprecipitated with Mab H7. Bound antigen was acid-eluted in citric acid, denatured and examined by SDS-PAGE followed by protein stain (A) and immunoblotting with N9 (B) or H7 (data not shown; each antibody gave indistinguishable blotting patterns).

Figures 10A-B show the protein and immunoblot profiles, respectively, of the specifically (H7) and nonspecifically associated (K16) proteins obtained from immunoprecipitation of detergent-solubilized degranulated plasma membranes. From the protein stain (Figure 10A), the 16 kD antigen present exclusively in the H7 eluate corresponds in relative molecular weight to the antigen isolated from membrane preparations made from both resting (Figures 2, 6) and fMLF-activated populations (Figures 7, 8). An additional 14 kD band is found in eluate of H7 suggested plasma membrane isolation of the free pro/cathelin domain of hCap. The presence of a band of similar molecular weight in K16 eluates suggested nonspecific association of lysozyme as indicated above. Immunoblots performed with Mab H7 on both H7 and K16 eluates revealed the 16 kD form to be the only reactive species of hCap present in H7 eluates and thus confirmed the nonspecific association of the 14 kD band with the H7 affinity matrix (Figure 10B). This latter band was identified as the nonspecific contaminant, lysozyme, in similar immunoprecipitation assays employing detergent-solubilized cellular homogenates from nonactivated neutrophil populations (see above).

Cell-Surface Binding Properties

The capacity of hCap to associate with activated neutrophil plasma membranes (Figures 7-10) suggests two possible mechanisms of hCap-membrane interaction. For example, hCap could be structurally modified in the extracellular milieu following its exocytosis so that it acquires the ability to insert into lipid bilayers. Alternatively, hCap-membrane interactions may result from recognition with a membrane-bound component

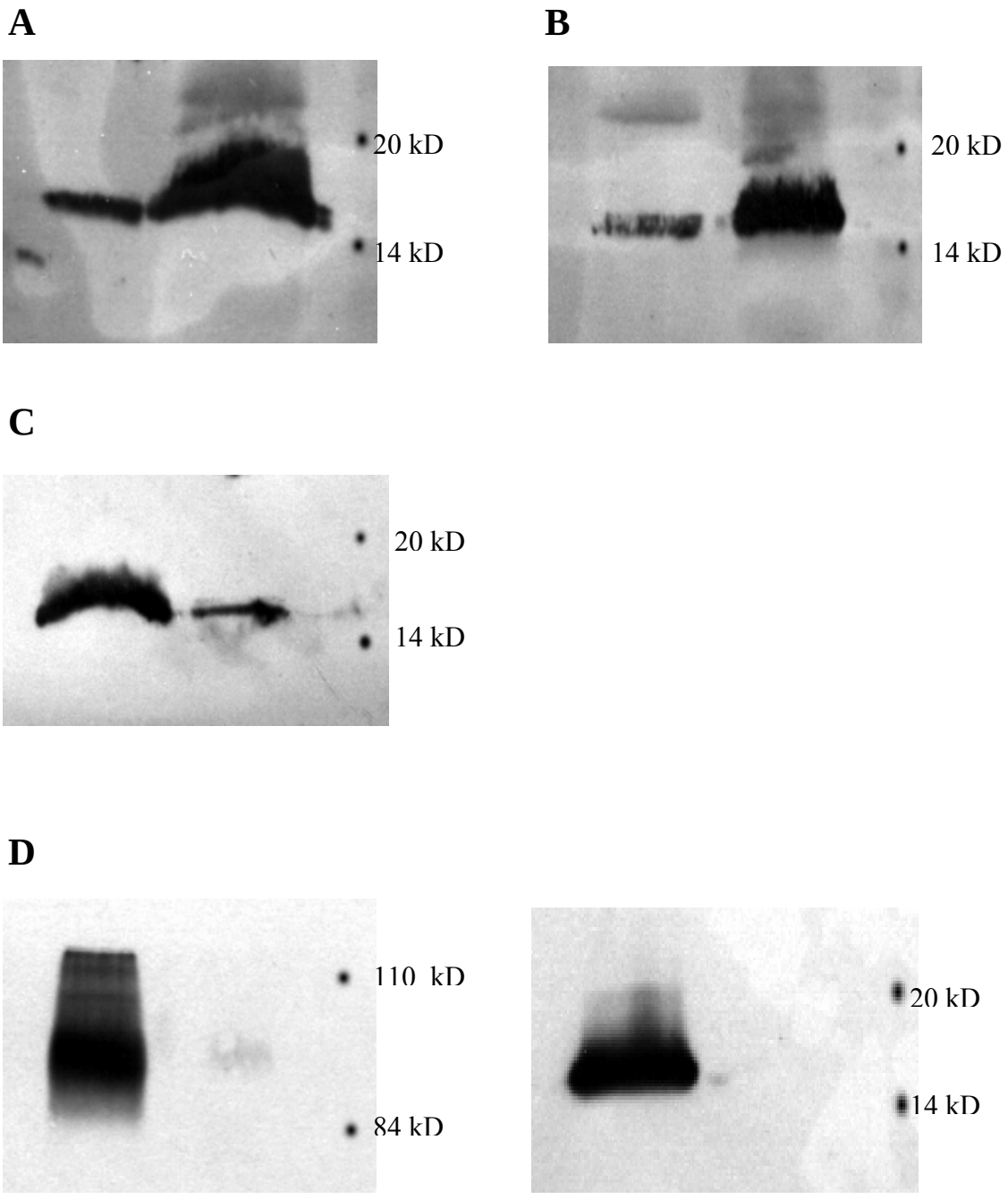


Figure 11. Surface membrane interactions of the 16 kD N9/H7 are salt resistant, sodium hydroxide sensitive, and partition hydrophobically into detergent-phase following membrane solubilization and clouding with Triton X-114. Degranulated neutrophil plasma membranes were isolated as described in Materials and Methods, washed in either

(Figure 11 –continued) DPBS (A), 1 M NaCl (B), or 10 mM NaOH (C) and analyzed by immunoblot with Mab H7. Each film depicts antigen content in the wash supernatant (left) and membrane (right) fractions. D) Degranulated membranes were solubilized in TritonX-114 at 4C. The detergent soluble fraction was then extracted by clouding and sedimentation at elevated temperatures (see Materials and Methods), and both detergent pellet (left lane) and hydrophilic (supernatant; right lane) phases were immunoblotted with Mab H7. The film on the left-hand-side of (D) shows the detergent partitioning of transmembrane protein control (gp 91^{phox}). The film on the right-hand-side of (D) depicts the detergent partitioning of the N9/H7 16 kD antigen.

that is either progressively upregulated to activated membranes or constitutively present but incapable of binding hCap until activation-related changes occur in receptor structure/function. We attempted to distinguish between these possibilities by determining the relative strength of hCap-membrane association. Treatment of purified plasma membrane fractions with solutions of high pH or ionic strength selectively eliminates peripheral membrane proteins (Steck and Yu, 1973;Luna et al., 1981). We applied these methods to examine the properties of hCap association with activated plasma membranes. Plasma membranes from degranulated neutrophils were prepared by N_2 cavitation of cells followed by high-speed sedimentation of post-nuclear supernatants to isolate plasma membrane-enriched pellet fractions. Membrane pellets were resuspended in buffer solutions containing either high salt or high pH, and the relative content of hCap present membrane pellet and supernatant fractions determined by immunoblot analysis. An apparent heterogeneity in the membrane binding capacity of hCap was indicated by the partial loss of surface hCap after resuspension in DPBS (Figure 11A). In contrast, membrane pellets further treated with 1M NaCl retained 70-85% of their original hCap complement, suggesting that a subset of hCap-plasma

membrane interactions are due to either peripheral, high affinity interactions or hydrophobic insertion into bilayers (Figure 11B). When membrane pellets were serially treated by resuspension in 1M NaCl solution followed by resuspension in 10 mM NaOH solution, the resulting membranes were nearly devoid of surface hCap (Figure 11C). This salt-stable, pH-sensitive interaction of surface hCap appear inconsistent with expected properties conferred by bilayer insertion (which should be unaffected by high pH). It cannot be ruled out that possible pH-related structural changes in native hCap may occur that alter its capacity for membrane insertion. In addition, interactions involving the high-affinity peripheral association of hCap with one or more integral membrane protein families are also possible.

Further evidence indicating the relative stability of hCap-plasma membrane association was obtained by subjecting purified plasma membrane fractions from fMLF-stimulated, dhCB-pretreated fully degranulated neutrophils to a Triton X-114 phase shift assay (Borregaard and Tauber, 1984). In this assay, transmembrane proteins partition into a hydrophobic, detergent-enriched fraction, while peripheral proteins partition into a detergent-depleted, aqueous fraction. The membrane fractions examined in this assay were pre-washed in 1 M NaCl to eliminate low affinity hCap interactions. The resulting salt-resistant fraction of membrane-bound hCap was found exclusively within the hydrophobic, detergent-enriched phase (Figure 11D, right), indicating that hCap-membrane interactions may result from a hydrophobic association with the membrane bilayer. These findings could explain the resistance of surface-bound hCap to high salt

and suggests that the pH sensitivity of hCap surface binding observed above may be due to conformational changes in hCap structure. Such alterations may underlie its capacity to interact with membrane bilayer or, instead, promote a very tight interaction with membrane proteins such as is found with actin (Luna et al., 1981).

Discussion

Data reported here describe the characterization of the two hCap-specific monoclonal antibodies, N9 and H7, and novel properties of hCap localization. The antibodies were developed using neutrophil cellular membranes as immunogen and specifically recognize a single ~16 kD band present in immunoblots of SDS-PAGE separated membrane material. N-terminal sequence analysis identified hCap as the N9/H7 antigen, and mass analysis of trypsin-digested antigen using MALDI mass spectrometry resulted in sequence coverages of 55% and 65% for the N-terminal pro/cathelin and C-terminal cationic domains, respectively. Structural analysis of N9 and H7 epitopes by phage display yielded the five amino acid consensus sequence, NARGZ, and corresponded to a motif located in the C-terminal region of the hCap pro/cathelicidin domain. Subsequent studies employing isopycnic sucrose density sedimentation of post-nuclear fractions from non-stimulated neutrophil populations revealed antigen co-enrichment with the specific granule marker, lactoferrin, and thus consistent with specific granule localization of neutrophil hCap. When antigen-localization properties were examined in greater detail, a novel capacity for antigen association with plasma

membranes from fMLF-stimulated neutrophils was found that was substantially enhanced on intact surfaces of fully degranulated neutrophils. This association was confirmed by immunoprecipitation of the ~16 kD hCap from purified plasma membranes from fMLF-stimulated and fully degranulated neutrophils and peptide mass mapping of the trypsin-digested antigen MALDI mass spectrometry. We conclude that the major fraction of surface-bound hCap is in high affinity association on the basis of (i) the stability of surface-hCap to 1M NaCl washes, (ii) the partitioning of detergent-solubilized surface-hCap complexes into a Triton X-114-enriched detergent phase, and (iii) the differential extraction of such complexes following treatment of membranes with 10 mM NaOH. Considered together, these data definitively show that N9 and H7 Mabs specifically recognize the native hCap of neutrophils and that neutrophil hCap, following its release from specific granules, tightly interacts with activated plasma membranes.

The five amino acid consensus sequence, NARGZ, obtained in this study identified residues 115, 117-119 and 121 in hCap primary structure with the sequence, ^{115}NXARGXZ^{121}, where X and Z indicate omitted and aromatic amino acids, respectively. The organization of this motif in primary structure has certain structural implications. For example, the discrete discontinuities in epitope sequence relative to the phage consensus are consistent with a highly condensed spatial geometry on the protein surface. This is supported by a previous study using phage display to map the epitope of an F-actin polyclonal antibody with molecular modeling (Jesaitis et al., 1999). It showed spatial proximity among residues on the surface of actin that were continuous in the

consensus motif but discontinuous in primary structure by single amino acid-hops. Further insights into the spatial geometry of the N9/H7 epitope as well as potential functional implications of N9/H7-hCap interaction can be inferred from a recent structural study of the porcine cathelicidin, protegrin-3, in which the crystal structure of the pro/cathelin domain was solved at 2.2 Å resolution (Sanchez et al., 2002). The pro/cathelin sequences of mammalian cathelicidins share 60% sequence identity (Bals and Wilson, 2003), making results from such studies relevant to the findings presented here. This structure included an 18-residue N-terminal helix cradled within a four-stranded (β1-4) anti-parallel β-sheet, with the β-chains linked together by an appending domain and two disordered loops. Residues 115-121 contribute to a solvent-exposed, 10-residue disordered loop (L2) linking the β3 and β4 domains. Additional modeling with the protegrin-1 antimicrobial peptide, a two-stranded β-sheet structure previously established by NMR (Aumelas et al., 1996), suggested the peptide anchored within the β-core cradle of the pro/cathelin domain, adjacent the N-terminal helix, and partially stabilized there by electrostatic interactions with residue 118 of L2. In fact, this model predicted that the majority of residues composing the peptide-pro/cathelin interface were from L2 and β4 domains. The possible importance of the N9/H7 consensus region in stabilization of cationic peptide, considered together with its linear proximity in primary structure to the protease 3 cleavage site at residue 132, suggests that N9/H7-hCap interactions could prevent or impair functional conversion of hCap.

Additional significance of the N9/H7 consensus region is indicated by recent studies examining the nature of protein-protein interfaces

(Clackson and Wells, 1995;Braisted and Wells, 1996;Bogan and Thorn, 1998;Clackson et al., 1998). These studies suggest a general molecular basis for the specificity and stabilization of antigen-antibody interactions that involves “hot spots,” which form the geometric center of and confer stability to protein-protein interaction sites and which principally contain tyrosine, tryptophan and arginine residues. All three of these amino acids are included in the shared N9/H7 epitope sequence and could indicate this region of hCap structure is one such energetically preferred site of intermolecular interaction.

Interestingly, the molecular weight of the ~16 kD antigen recognized by N9 and H7 monoclonals differs slightly from the ~18 kD native form of hCap identified in several previous reports (Sorensen et al., 1997a;Bals et al., 1998;Bals et al., 1999a;Malm et al., 2000;Andersson et al., 2002). Mass analysis revealed that the 16 kD antigen structure consisted of both pro- and active-domains with a respective sequence coverage of 55% and 65% (Table 3). This minor discrepancy in the relative molecular mass of hCap would therefore appear to be due to inherent quantitative limitations of SDS-PAGE in estimating molecular mass (Hjelmeland et al., 1979;Hjelmeland and Chrambach, 1981;Hjelmeland and Chrambach, 1984;Sallantin et al., 1990).

The resistance of hCap-plasma membrane to high molar salt coupled with its hydrophobic partitioning during solubilization of membranes in Triton X-114 detergent is consistent with insertion of hCap into the membrane bilayer (Bordier, 1981). On the other hand, the extraction of surface-hCap in 10 mM NaOH-washed plasma membranes could indicate that hCap surface association is mediated by high affinity interaction with one or more membrane protein receptors. The amino acid composition and structural

organization of hCap may offer further insight into the nature of hCap-membrane association. According to structural data obtained from the porcine cathelicidin, protegrin-3, the C-terminus of native hCap is likely sequestered within the more extensive N-terminal pro/cathelicidin domain. The primary sequence of the N-terminal region of hCap is sufficiently hydrophobic and could potentially confer membrane anchorage (Figures 3A-C).

C	C	C	C	C	C	C	C	C	C	C	H	H	H	H	H	H	H	H	H	H	H	C	C
M	K	K	Q	R	D	F	H	S	L	G	R	W	S	L	V	L	L	L	L	G	L	V	M	P

C	C	C	C	C	C	C	C	C	H	H	H	H	H	H	H	H	H	H	H	H	C	C	C	C
L	A	I	I	A	Q	V	L	S	Y	K	E	A	V	L	R	A	I	D	G	I	N	Q	R	S

Figure 12. Predicted secondary structure of first N-terminal 50 amino acids of the pro/cathelin domain. Structural prediction using Web-based 3D-PSSM software modeled on the porcine cathelicidin, protegrin-3. Top row: H = α-helical region; C = β sheet. Bottom row: single letter amino acid code.

Figure 12 shows the predicted secondary structure of the first 50 amino acids of the pro/cathelin domain modeled on the crystal structure of protegrin-3 using 3D-PSSM software (see Materials and Methods). The predicted structure indicates a potential alpha helical arrangement formed by largely hydrophobic residues (12-23) at the N-terminus of the pro/cathelin domain that could facilitate bilayer association of the native hCap.

On the other hand, the proposed mechanism of hCap microbicidal action relies, in part, on the solubility of the native form and the relative hydrophobicity of its active peptide, LL-37. More specifically, membrane insertion activity of the native form of hCap could severely limit the antimicrobial potential of the active LL-37 antibiotic peptide at extravascular sites of infection by concentrating precursor molecules at sites of their initial secretion. This would in turn predispose host cells bearing native hCap to the lytic action of LL-37 following peptide cleavage. Indeed, the marked sensitivity of surface-hCap to the more stringent conditions imposed by membrane resuspension in 10 mM NaOH would suggest that hCap-membrane interactions are due to a high affinity association with membrane protein rather than bilayer insertion. Accordingly, pH-induced changes in the conformation of protein receptors could result in a loss of receptor-ligand interaction. This explanation is consistent with the presumed high solubility of the native hCap within both the extracellular milieu and in the matrix of neutrophil specific granules.

Many previous reports have documented the ability of human neutrophils to stably occupy functionally diverse cellular activation states (Sengelov et al., 1995);(Dewald et al., 1982;Kjeldsen et al., 1992;Kjeldsen et al., 1993;Sengelov et al., 1993;Borregaard et al., 1994;Sengelov et al., 1994;Faldt et al., 2001;Lollike et al., 2002;Bylund et al., 2002;Kaldi et al., 2002;Tapper et al., 2002). This phenomenon is associated with a graded, as opposed to an all-or-nothing, cellular response to microenvironmental stimuli that in turn suggests a functional basis for the cell surface-localization of native hCap observed in this study (Figs 7-10). The positive correlation

between cell surface-adherence of hCap and neutrophil activation reported here could optimally position native hCap at the neutrophil-bacterial cell interface. Such proximity could confer a spatial specificity on hCap activation by extracellular proteases that directs antimicrobial action toward juxtaposed bacterial membranes while simultaneously minimizing damage to host tissues. Moreover, plasma membrane-localized hCap would be ideally situated for the *in situ* killing and degradation of microbes before, during, and after their phagocytic uptake. Prior to the publication of this study, reports documenting hCap expression on cell surface membranes were confined to germ line cells, which can be coated with as many as 6.6 million copies of hCap per cell (Malm et al., 2000;Andersson et al., 2002). Although a function for hCap in reproductive biology has not yet been identified, the possibility that the active maintenance of native hCap at the cellular-microenvironmental interface could supplement the defensive capacity of such cells has been postulated (Lehrer and Ganz, 2002). Given that hCap is likely soluble within specific granules (Cowland et al., 1995;Sorensen et al., 1997a;Sorensen et al., 1997b;Bulow et al., 2002) and that as much as 25% of this cellular fraction can be lost during fMLF-stimulation in the absence of phagocytosis (Sengelov et al., 1993), significant quantities of hCap may be released during the extravasation and subsequent transit of neutrophils into infected tissues. Progressive cell surface-association of native hCap could therefore serve as a novel mechanism to maximize the recovery and transport of soluble hCap into sites of microbial infection, as well as enhance the efficacy and insure the specificity of hCap-antimicrobial action.

LITERATURE CITED

Afeltra A, Caccavo D, Ferri GM, Addessi MA, De Rosa FG, Amoroso A, and Bonomo L. 1997. Expression of lactoferrin on human granulocytes: analysis with polyclonal and monoclonal antibodies. Clin Exp Immunol 109:279-285.

Ambruso DR, Bentwood B, Henson PM, and Johnston RB, Jr. 1984. Oxidative metabolism of cord blood neutrophils: relationship to content and degranulation of cytoplasmic granules. Pediatr Res 18:1148-1153.

Andersson E, Sorensen OE, Frohm B, Borregaard N, Egesten A, and Malm J. 2002. Isolation of human cationic antimicrobial protein-18 from seminal plasma and its association with prostasomes. Hum Reprod 17:2529-2534.

Aumelas A, Mangoni M, Roumestand C, Chiche L, Despaux E, Grassy G, Calas B, and Chavanieu A. 1996. Synthesis and solution structure of the antimicrobial peptide protegrin-1. Eur J Biochem 237:575-583.

Bals R, Wang X, Zasloff M, and Wilson JM. 1998. The peptide antibiotic LL-37/hCAP-18 is expressed in epithelia of the human lung where it has broad antimicrobial activity at the airway surface. Proc Natl Acad Sci U S A 95:9541-9546.

Bals R, Weiner DJ, Meegalla RL, and Wilson JM. 1999b. Transfer of a cathelicidin peptide antibiotic gene restores bacterial killing in a cystic fibrosis xenograft model. J Clin Invest 103:1113-1117.

Bals R, Weiner DJ, Moscioni AD, Meegalla RL, and Wilson JM. 1999a. Augmentation of innate host defense by expression of a cathelicidin antimicrobial peptide. Infect Immun 67:6084-6089.

Bals R and Wilson JM. 2003. Cathelicidins--a family of multifunctional antimicrobial peptides. Cell Mol Life Sci 60:711-720.

Bogan AA and Thorn KS. 1998. Anatomy of hot spots in protein interfaces. J Mol Biol 280:1-9.

Bordier C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. J Biol Chem 256:1604-1607.

Borregaard N, Christensen L, Bejerrum OW, Birgens HS, and Clemmensen I. 1990. Identification of a highly mobilizable subset of human neutrophil intracellular vesicles that contains tetranectin and latent alkaline phosphatase. J Clin Invest 85:408-416.

Borregaard N, Kjeldsen L, Sengelov H, Diamond MS, Springer TA, Anderson HC, Kishimoto TK, and Bainton DF. 1994. Changes in subcellular localization and surface expression of L-selectin, alkaline phosphatase, and Mac-1 in human neutrophils during stimulation with inflammatory mediators. J Leukoc Biol 56:80-87.

Borregaard N and Tauber AI. 1984. Subcellular localization of the human neutrophil NADPH oxidase. b-Cytochrome and associated flavoprotein. J Biol Chem 259:47-52.

Boxer LA, Haak RA, Yang HH, Wolach JB, Whitcomb JA, Butterick CJ, and Baehner RL. 1982. Membrane-bound lactoferrin alters the surface properties of polymorphonuclear leukocytes. J Clin Invest 70:1049-1057.

Braisted AC and Wells JA. 1996. Minimizing a binding domain from protein A. Proc Natl Acad Sci U S A 93:5688-5692.

Bulow E, Bengtsson N, Calafat J, Gullberg U, and Olsson I. 2002. Sorting of neutrophil-specific granule protein human cathelicidin, hCAP-18, when constitutively expressed in myeloid cells. J Leukoc Biol 72:147-153.

Burritt JB, Bond CW, Doss KW, and Jesaitis AJ. 1996. Filamentous phage display of oligopeptide libraries. Anal Biochem 238:1-13.

Burritt JB, Foubert TR, Baniulis D, Lord CI, Taylor RM, Mills JS, Baughan TD, Roos D, Parkos CA, and Jesaitis AJ. 2003. Functional epitope on human neutrophil flavocytochrome b558. J Immunol 170:6082-6089.

Burritt JB, Quinn MT, Jutila MA, Bond CW, and Jesaitis AJ. 1995. Topological mapping of neutrophil cytochrome b epitopes with phage-display libraries. J Biol Chem 270:16974-16980.

Bylund J, Karlsson A, Boulay F, and Dahlgren C. 2002. Lipopolysaccharide-induced granule mobilization and priming of the neutrophil response to Helicobacter pylori peptide Hp(2-20), which activates formyl peptide receptor-like 1. Infect Immun 70:2908-2914.

Clackson T, Ultsch MH, Wells JA, and de Vos AM. 1998. Structural and functional analysis of the 1:1 growth hormone:receptor complex reveals the molecular basis for receptor affinity. J Mol Biol 277:1111-1128.

Clackson T and Wells JA. 1995. A hot spot of binding energy in a hormone-receptor interface. Science 267:383-386.

Cowland JB, Johnsen AH, and Borregaard N. 1995. hCAP-18, a cathelin/pro-bactenecin-like protein of human neutrophil specific granules. FEBS Lett 368:173-176.

Davidson DJ, Currie AJ, Reid GS, Bowdish DM, MacDonald KL, Ma RC, Hancock RE, and Speert DP. 2004. The cationic antimicrobial peptide LL-37 modulates dendritic cell differentiation and dendritic cell-induced T cell polarization. J Immunol 172:1146-1156.

De Y, Chen Q, Schmidt AP, Anderson GM, Wang JM, Wooters J, Oppenheim JJ, and Chertov O. 2000. LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. J Exp Med 192:1069-1074.

DeChatelet LR and Cooper MR. 1970. A modified procedure for the determination of leukocyte alkaline phosphatase. Biochem Med 4:61-68.

Dewald B, Bretz U, and Baggiolini M. 1982. Release of gelatinase from a novel secretory compartment of human neutrophils. J Clin Invest 70:518-525.

Di Nardo A, Vitiello A, and Gallo RL. 2003. Cutting edge: mast cell antimicrobial activity is mediated by expression of cathelicidin antimicrobial peptide. J Immunol 170:2274-2278.

Elssner A, Duncan M, Gavrilin M, and Wewers MD. 2004. A novel P2X7 receptor activator, the human cathelicidin-derived peptide LL37, induces IL-1 beta processing and release. J Immunol 172:4987-4994.

Faldt J, Dahlgren C, Ridell M, and Karlsson A. 2001. Priming of human neutrophils by mycobacterial lipoarabinomannans: role of granule mobilisation. Microbes Infect 3:1101-1109.

Frohm M, Agerberth B, Ahangari G, Stahle-Backdahl M, Liden S, Wigzell H, and Gudmundsson GH. 1997. The expression of the gene coding for the antibacterial peptide LL-37 is induced in human keratinocytes during inflammatory disorders. J Biol Chem 272:15258-15263.

Frohm NM, Sandstedt B, Sorensen O, Weber G, Borregaard N, and Stahle-Backdahl M. 1999. The human cationic antimicrobial protein (hCAP18), a peptide antibiotic, is widely expressed in human squamous epithelia and colocalizes with interleukin-6. Infect Immun 67:2561-2566.

Ganz T, Selsted ME, Szklarek D, Harwig SS, Daher K, Bainton DF, and Lehrer RI. 1985. Defensins. Natural peptide antibiotics of human neutrophils. J Clin Invest 76:1427-1435.

Heilborn JD, Nilsson MF, Kratz G, Weber G, Sorensen O, Borregaard N, and Stahle-Backdahl M. 2003. The cathelicidin anti-microbial peptide LL-37 is involved in re-epithelialization of human skin wounds and is lacking in chronic ulcer epithelium. J Invest Dermatol 120:379-389.

Henson PM and Oades ZG. 1975. Stimulation of human neutrophils by soluble and insoluble immunoglobulin aggregates. Secretion of granule constituents and increased oxidation of glucose. J Clin Invest 56:1053-1061.

Hjelmeland LM and Chrambach A. Electrophoresis and electrofocusing in detergent containing media: a discussion of basic concepts. Electrophoresis 2, 1-11. 1981.
Ref Type: Generic

Hjelmeland LM and Chrambach A. 1984. Solubilization of functional membrane proteins. Methods Enzymol 104:305-318.

Hjelmeland LM, Nebert DW, and Chrambach A. 1979. Electrofocusing of integral membrane proteins in mixtures of zwitterionic and nonionic detergents. Anal Biochem 95:201-208.

Islam D, Bandholtz L, Nilsson J, Wigzell H, Christensson B, Agerberth B, and Gudmundsson G. 2001. Downregulation of bactericidal peptides in enteric infections: a novel immune escape mechanism with bacterial DNA as a potential regulator. Nat Med 7:180-185.

Jesaitis AJ, Gizachew D, Dratz EA, Siemsen DW, Stone KC, and Burritt JB. 1999. Actin surface structure revealed by antibody imprints: evaluation of phage-display analysis of anti-actin antibodies. Protein Sci 8:760-770.

Kaldi K, Szeberenyi J, Rada BK, Kovacs P, Geiszt M, Mocsai A, and Ligeti E. 2002. Contribution of phopholipase D and a brefeldin A-sensitive ARF to chemoattractant-induced superoxide production and secretion of human neutrophils. J Leukoc Biol 71:695-700.

Kelley LA, MacCallum RM, and Sternberg MJ. 2000. Enhanced genome annotation using structural profiles in the program 3D-PSSM. J Mol Biol 299:499-520.

Kirikae T, Hirata M, Yamasu H, Kirikae F, Tamura H, Kayama F, Nakatsuka K, Yokochi T, and Nakano M. 1998. Protective effects of a human 18-kilodalton cationic antimicrobial protein (CAP18)-derived peptide against murine endotoxemia. Infect Immun 66:1861-1868.

Kjeldsen L, Bainton DF, Sengelov H, and Borregaard N. 1993. Structural and functional heterogeneity among peroxidase-negative granules in human neutrophils: identification of a distinct gelatinase-containing granule subset by combined immunocytochemistry and subcellular fractionation. Blood 82:3183-3191.

Kjeldsen L, Bjerrum OW, Askaa J, and Borregaard N. 1992. Subcellular localization and release of human neutrophil gelatinase, confirming the existence of separate gelatinase-containing granules. Biochem J 287 (Pt 2):603-610.

Koczulla R, von Degenfeld G, Kupatt C, Krotz F, Zahler S, Gloe T, Issbrucker K, Unterberger P, Zaiou M, Lebherz C, Karl A, Raake P, Pfosser A, Boekstegers P, Welsch U, Hiemstra PS, Vogelmeier C, Gallo RL, Clauss M, and Bals R. 2003. An angiogenic role for the human peptide antibiotic LL-37/hCAP-18. J Clin Invest 111:1665-1672.

Lehrer RI and Ganz T. 2002. Cathelicidins: a family of endogenous antimicrobial peptides. Curr Opin Hematol 9:18-22.

Lollike K, Lindau M, Calafat J, and Borregaard N. 2002. Compound exocytosis of granules in human neutrophils. J Leukoc Biol 71:973-980.

Luna EJ, Fowler VM, Swanson J, Branton D, and Taylor DL. 1981. A membrane cytoskeleton from Dictyostelium discoideum. I. Identification and partial characterization of an actin-binding activity. J Cell Biol 88:396-409.

Malm J, Sorensen O, Persson T, Frohm-Nilsson M, Johansson B, Bjartell A, Lilja H, Stahle-Backdahl M, Borregaard N, and Egesten A. 2000. The human cationic antimicrobial protein (hCAP-18) is expressed in the epithelium of human epididymis, is present in seminal plasma at high concentrations, and is attached to spermatozoa. Infect Immun 68:4297-4302.

Manev V, Maneva A, and Sirakov L. 1998. Effect of lactoferrin on the phagocytic activity of polymorphonuclear leucocytes isolated from blood of patients with autoimmune diseases and Staphylococcus aureus allergy. Adv Exp Med Biol 443:321-330.

Mukherjee G, Quinn MT, Linner JG, and Jesaitis AJ. 1994. Remodeling of the plasma membrane after stimulation of neutrophils with f-Met-Leu-Phe and dihydrocytochalasin B: identification of membrane subdomains containing NADPH oxidase activity. J Leukoc Biol 55:685-694.

Murakami M, Lopez-Garcia B, Braff M, Dorschner RA, and Gallo RL. 2004. Postsecretory processing generates multiple cathelicidins for enhanced topical antimicrobial defense. J Immunol 172:3070-3077.

Mutasa HC. 1989. Combination of diaminobenzidine staining and immunogold labeling: a novel technical approach to identify lysozyme in human neutrophil cells. Eur J Cell Biol 49:319-325.

Niyonsaba F, Iwabuchi K, Someya A, Hirata M, Matsuda H, Ogawa H, and Nagaoka I. 2002. A cathelicidin family of human antibacterial peptide LL-37 induces mast cell chemotaxis. Immunology 106:20-26.

Niyonsaba F, Someya A, Hirata M, Ogawa H, and Nagaoka I. 2001. Evaluation of the effects of peptide antibiotics human beta-defensins-1/-2 and LL-37 on histamine release and prostaglandin D(2) production from mast cells. Eur J Immunol 31:1066-1075.

Nizet V, Ohtake T, Lauth X, Trowbridge J, Rudisill J, Dorschner RA, Pestonjamasp V, Piraino J, Huttner K, and Gallo RL. 2001. Innate antimicrobial peptide protects the skin from invasive bacterial infection. Nature 414:454-457.

Ong PY, Ohtake T, Brandt C, Strickland I, Boguniewicz M, Ganz T, Gallo RL, and Leung DY. 2002. Endogenous antimicrobial peptides and skin infections in atopic dermatitis. N Engl J Med 347:1151-1160.

Oren Z, Lerman JC, Gudmundsson GH, Agerberth B, and Shai Y. 1999. Structure and organization of the human antimicrobial peptide LL-37 in phospholipid membranes: relevance to the molecular basis for its non-cell-selective activity. Biochem J 341 (Pt 3):501-513.

Oseas R, Yang HH, Baehner RL, and Boxer LA. 1981. Lactoferrin: a promoter of polymorphonuclear leukocyte adhesiveness. Blood 57:939-945.

Parkos CA, Allen RA, Cochrane CG, and Jesaitis AJ. 1987. Purified cytochrome b from human granulocyte plasma membrane is comprised of two polypeptides with relative molecular weights of 91,000 and 22,000. J Clin Invest 80:732-742.

Putsep K, Carlsson G, Boman HG, and Andersson M. 2002. Deficiency of antibacterial peptides in patients with morbus Kostmann: an observation study. Lancet 360:1144-1149.

Sallantin M, Huet JC, Demarteau C, and Pernollet JC. 1990. Reassessment of commercially available molecular weight standards for peptide sodium dodecyl sulfate-polyacrylamide gel electrophoresis using electroblotting and microsequencing. Electrophoresis 11:34-36.

Sanchez JF, Hoh F, Strub MP, Aumelas A, and Dumas C. 2002. Structure of the cathelicidin motif of protegrin-3 precursor: structural insights into the activation mechanism of an antimicrobial protein. Structure (Camb) 10:1363-1370.

Scott MG, Davidson DJ, Gold MR, Bowdish D, and Hancock RE. 2002. The human antimicrobial peptide LL-37 is a multifunctional modulator of innate immune responses. J Immunol 169:3883-3891.

Sengelov H, Boulay F, Kjeldsen L, and Borregaard N. 1994. Subcellular localization and translocation of the receptor for N-formylmethionyl-leucyl-phenylalanine in human neutrophils. Biochem J 299 (Pt 2):473-479.

Sengelov H, Follin P, Kjeldsen L, Lollike K, Dahlgren C, and Borregaard N. 1995. Mobilization of granules and secretory vesicles during in vivo exudation of human neutrophils. J Immunol 154:4157-4165.

Sengelov H, Kjeldsen L, and Borregaard N. 1993. Control of exocytosis in early neutrophil activation. J Immunol 150:1535-1543.

Shai Y. 2002. Mode of action of membrane active antimicrobial peptides. Biopolymers 66:236-248.

Smith GP and Scott JK. 1993. Libraries of peptides and proteins displayed on filamentous phage. Methods Enzymol 217:228-257.

Sorensen O, Arnljots K, Cowland JB, Bainton DF, and Borregaard N. 1997b. The human antibacterial cathelicidin, hCAP-18, is synthesized in myelocytes and metamyelocytes and localized to specific granules in neutrophils. Blood 90:2796-2803.

Sorensen O, Cowland JB, Askaa J, and Borregaard N. 1997a. An ELISA for hCAP-18, the cathelicidin present in human neutrophils and plasma. J Immunol Methods 206:53-59.

Sorensen OE, Follin P, Johnsen AH, Calafat J, Tjabringa GS, Hiemstra PS, and Borregaard N. 2001. Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3. Blood 97:3951-3959.

Sorensen OE, Gram L, Johnsen AH, Andersson E, Bangsboll S, Tjabringa GS, Hiemstra PS, Malm J, Egesten A, and Borregaard N. 2003. Processing of seminal plasma hCAP-18 to ALL-38 by gastricsin: a novel mechanism of generating antimicrobial peptides in vagina. J Biol Chem 278:28540-28546.

Steck TL and Yu J. 1973. Selective solubilization of proteins from red blood cell membranes by protein perturbants. J Supramol Struct 1:220-232.

Stie J and Jesaitis AJ. Preparation of Secretory Vesicle-Free Plasma Membranes by Isopycnic Sucrose Gradient Fractionation of Neutrophils by the Gelatin Method. Cytotechnology 46[Numbers 2-3], 109-122. 2004.
Ref Type: Generic

Swain SD, Jutila KL, and Quinn MT. 2000. Cell-surface lactoferrin as a marker for degranulation of specific granules in bovine neutrophils. Am J Vet Res 61:29-37.

Tapper H, Karlsson A, Morgelin M, Flodgaard H, and Herwald H. 2002. Secretion of heparin-binding protein from human neutrophils is determined by its localization in azurophilic granules and secretory vesicles. Blood 99:1785-1793.

Tjabringa GS, Aarbiou J, Ninaber DK, Drijfhout JW, Sorensen OE, Borregaard N, Rabe KF, and Hiemstra PS. 2003. The antimicrobial peptide LL-37 activates innate immunity at the airway epithelial surface by transactivation of the epidermal growth factor receptor. J Immunol 171:6690-6696.

Tomasinsig L and Zanetti M. 2005. The cathelicidins--structure, function and evolution. Curr Protein Pept Sci 6:23-34.

Tossi A, Sandri L, and Giangaspero A. 2000. Amphipathic, alpha-helical antimicrobial peptides. Biopolymers 55:4-30.

Zanetti M, Gennaro R, and Romeo D. 1995. Cathelicidins: a novel protein family with a common proregion and a variable C-terminal antimicrobial domain. FEBS Lett 374:1-5.

Zasloff M. 2002. Antimicrobial peptides of multicellular organisms. Nature 415:389-395.

CHAPTER 5

ISOLATION OF CHOLESTEROL-DEPENDENT CANDIDA GLABRATA FROM CLINICAL SPECIMENS

Abstract

In the past few years, we have detected in the United Kingdom and in the United States several isolates of *Candida glabrata* that grew poorly unless bile was available. Cholesterol, a component of bile, stimulated equivalent growth of the bile-dependent isolates. The bile-dependent C. glabrata isolates appeared resistant to amphotericin B, but their resistance to fluconazole was unclear. These results demonstrate that occasional isolates of *Candida glabrata* require cholesterol to grow, they may not be detected in specimens set up on standard primary plating media, and they may be difficult to eradicate in patients with antifungal agents directed against ergosterol and its synthetic pathway.

Introduction

Candida albicans has historically been responsible for most forms of candidiasis. *Candida glabrata* has become a more frequent agent of urinary tract infection, presumably because of its greater resistance to fluconazole and other azoles compared with *Candida albicans* (Hazen et al., 2003;Pfaller et al., 2002). Within the clinical laboratory, detection of *Candida glabrata* as an agent of urinary tract infection may not occur because some strains may require more than 48 hours to form detectable colonies

on routine urine screens, especially if the patient is being treated with antifungals. Here, we describe our initial observation and subsequent studies regarding bile-dependent and bile-enhanced growth of *Candida glabrata* isolates from urine, blood, and nasopharyngeal specimens.

Case Report

A 67-year-old man in the United Kingdom underwent a bilateral nephrectomy and one year later, a cadaveric renal transplant. Seven years post-transplant, he began to experience urinary tract problems. Beginning one month later and persisting for five months, the patient's urine specimens frequently contained white blood cells and yeasts, which were isolated on Sabouraud's glucose agar (SGA) and were germ tube-negative (i.e., presumptively non-*Candida albicans*). The patient received a two-week course of fluconazole (50 mg/day) during third month following initial symptoms. In the fifth month, he received amphotericin B (1 mg/[kg/day]$^{-1}$) but was switched to itraconazole (400 mg/day for 16 days. Subsequent urine cultures revealed white blood cells and few yeasts, but the yeasts did not grow on SGA. However, two specimen grew yeasts during this time; isolate 73246 grew only on modified Leeming's medium (LM), which facilitates growth of *Malassezia* sp., and isolate L999 grew on SGA. The patient received another course of fluconazole but died two months later from underlying ischemic heart disease.

Subsequent studies demonstrated that, when added to SGA, ox bile, a component of modified LM agar, encouraged the same rate of growth of strain 73246 as obtained on

the modified LM agar. Other lipid components of modified LM agar, Tween 60 (polyoxyethylenesorbitan monostearate), glycerol, glycerol monostearate, and full-fat mild produced small colonies only after one week. When isolate 73246 was subcultured onto SGA containing one of the following sterols suspended in ethanol, cholesterol, lanosterol, and ergosterol, better growth of isolate 73246 occurred on the cholesterol-containing medium.

The morphology of both isolates was consistent with *Candida glabrata*. Isolate L999 was identified as Candida glabrata with the API-20C (bioMerieux, Marcy l' Etoile, France). Isolate 73246 could not be identified in this manner because it failed to grow. The two yeast isolates were determined to be similar by karyotyping and restriction endonuclease analysis as described by Arif et al. (1996). Confirmation that the isolates were Candida glabrata was achieved by amplification of the intergenic spacer regions of rDNA (Williams et al., 1995) followed by digestion of the PCR product with the restriction endonuclease TaqI (Pharmacia LKB, Uppsala, Sweden) and separation of the fragments on five percent acrylamide gels. Hence, isolate 73246 appears to be nutritional variant of *Candida glabrata* in that it requires bile for growth.

Subsequent Studies

Detection of Additional Bile-Dependent Candida Glabrata

In subsequent studies in the United States, additional bile-dependent variants of *Candida glabrata* were obtained. Urine and other specimens in which yeasts were seen but did not grow on routine culture or showed minute colonies on MacConkey agar were

inoculated onto SGA plates on which a bile disk (15 mg/disk, Remel, Lenexa, KS) was placed. In some cases, minute colonies on routine culture were seen, microscopy of the colonies revealed yeasts, but the yeasts failed to grow upon subculture onto SGA unless a bile disk was present. As a result of these efforts, additional phenotypically stable bile-dependent and bile-enhanced *Candida glabrata* isolates were obtained. Not all isolates were obtained from urine specimens, as we recovered a single isolate from a nasopharyngeal specimen. During one seven-month period, we isolated bile-dependent isolates from six patients. We also isolated several Candida glabrata isolates that initially appeared bile-dependent or with bile-enhanced growth, but upon subsequent subcultures, the isolates recovered bile independence, presumable because of selection. One of these isolates was obtained from a blood culture. All isolates were confirmed as Candida glabrata based on microscopic morphology and rapid trehalose assimilation (National Committee of Clinical Laboratory Standards, 2002).

To assess whether routine culture of urine should include an agar medium specifically designed to support growth of bile-dependent yeasts and to obtain an estimate of how common initially bile-dependent isolates are present in urine specimens, 169 urine specimens were collected over a one-month period and inoculated using a standard loop onto SGA plates containing 0.4% ox bile (SGA-B) and onto sheep blood agar (BA) plates within 24 hours after collection. The plates were incubated at 35C and assessed daily for 10 days. No attempt was made in this survey to determine the clinical significance of these isolates or to identify them. Thirty-seven specimens (21.9%) grew yeasts. Yeasts were recovered from 30 specimens (81.1%) on culture by 24 hours,

whereas six of the remaining seven specimens were culture positive at 48 hours. Yeasts were recovered only on SGA-B from four of the 37 positive specimens [(10.8%) (Table 1)], suggesting these isolates may require bile for growth. Two of these were not positive until 48 hours. These four isolates reverted to bile independence upon further subculture. Therefore, no stable bile-dependent isolates were recovered during this limited 1-month survey. The result, however, indicates that these isolates would have not been recovered from the specimens unless the bile-containing medium was included. For seven specimens (18.9%), yeasts were detected only on the BA plate, suggesting that some yeasts may be inhibited by bile (six of these were detected on BA at 24 hours and one at 48 hours).

Table 1: Detection of Bile-Dependent Yeasts from 169 Urine Specimens Collected Over a One-Month Period

Medium	Number of specimens (% of total positive specimens)[a]	Number of specimens requiring 48 hours or more for recovery of yeasts
BA and SGA-B	26 (70.2)	3 (48 hours)
BA only	7 (18.9)	1 (48 hours), 1 (96 hours)
SGA-B only	4 (10.8)	2 (48 hours)

[a] Yeasts were recovered from 37 specimens (21.9%).

Effect of Lipid Supplementation

Various lipids (1 mmol/L in water), including cholesterol (a component of bile), Tween 20 (polyoxyethylenesorbitan monolaurate) and Tween 40 (polyoxyethylenesorbitan monopalmitate) were tested for their ability to stimulate growth of the bile-dependent isolates using a pour-plate technique in which the lipids were added to wells but in the agar plates. At 24 hours (30 °C), no growth was obtained

Table 2: Effect of cholesterol, Tween 20, and Tween 40 (1 mmol/L) on growth of bile-dependent *Candida glabrata* isolates

Component	Number of positive/bile-dependent isolates tested
Bile	6/6
Cholesterol	0/6
Tween 20	0/6 [a]
Tween 40	0/6 [a]
Tween 20 + cholesterol	5/6

[a] A halo of slight growth was noted, suggesting a particular concentration of the agents may support growth

with any of the lipids alone, but the combination of cholesterol and Tween 20 stimulated growth (Table 2), presumably because cholesterol is more soluble in Tween 20 than

water. Cholesterol- saturated water mixed with various concentrations of Tween 20 (1.25%, 2.5%, and 5% in the formation of a distinct zone of growth for each Tween 20 concentration and the zone diameters directly related to the concentration of Tween 20. These results confirm that cholesterol is a component of bile that supports growth of nutritionally variant isolates.

Minimal Inhibitory Concentrations

Four bile-dependent isolates of *Candida glabrata* were tested along with two quality control strains, *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019, in a macrobroth dilution assay (National Committee of Clinical Laboratory Standards, 1997). We also tested a bile-independent isolate of *Candida glabrata* and one bile-enhanced isolate. Ox bile extract (0.4%) (ICN Biomedicals, Aurora, OH) was added to the test medium, RPMI 1640. For *Candida parapsilosis*, the addition of bile caused essentially no change in the apparent minimal inhibitory concentrations of fluconazole and amphotericin B. For *Candida krusei*, the presence of bile increased the minimal inhibitory concentration of fluconazole from 16 to 64 μg/mL, and the minimal inhibitory concentration of amphotericin B from 0.25 to 4 μg/mL. For the bile-independent *Candida glabrata*, bile had no effect on the minimal inhibitory concentration of amphotericin B (which was one dilution higher than that of *Candida krusei*, or 0.5 μg/mL) but changed the minimal inhibitory concentration of fluconazole form 0.125 to 4 μg/mL. The four bile-dependent isolates and one bile-enhanced isolate of *Candida glabrata* appeared

resistant to both amphotericin B and fluconazole, with minimal inhibitory concentration values exceeding 16 μg/mL and 64 μg/mL, respectively, although given the affect of bile on fluconazole susceptibility of bile-independent *Candida glabrata*, the minimal inhibitory concentration values obtained for fluconazole may be lower. These results suggest antifungal agents that target ergosterol and its synthetic pathway may be ineffective against bile-dependent isolates of *Candida glabrata*.

In summery, our results indicate that the diagnosis of urinary tract infections caused by *Candida glabrata* may be missed because of lack of growth of cholesterol-dependent variants on standard primary culture media. Moreover, these variants may present an antifungal management challenge. It is unclear what patient population is most likely to harbor cholesterol-dependent *Candida glabrata*. Exposure to azoles does not appear to be required to select for these strains, as some of the patients from whom bile-dependent isolates were obtained had not recently been exposed to antifungal agents.

LITERATURE CITED

Hazen KC, Baron EJ, Colombo AL, Girmenia C, Sanchez-Sousa A, del Palacio A, de Bedout C, and Gibbs DL. 2003. Comparison of the susceptibilities of Candida spp. to fluconazole and voriconazole in a 4-year global evaluation using disk diffusion. J Clin Microbiol 41:5623-5632.

National Committee of Clinical Laboratory Standards. 1997. Reference Method for Broth Dilution Antifungal Susceptability Testing of Yeasts: Approved Standard NCCLS document M27-A. Wayne, (PA): National Committee of Clinical Laboratory Standards31-32.

National Committee of Clinical Laboratory Standards. 2002. Abbreviated Identification of Bacteria and Yeasts: Approved Guideline NCCLS document M-35A. Wayne, (PA): National Committee of Clinical Laboratory Standards1-36.

Pfaller MA, Messer SA, Hollis RJ, Jones RN, and Diekema DJ. 2002. In vitro activities of ravuconazole and voriconazole compared with those of four approved systemic antifungal agents against 6,970 clinical isolates of Candida spp. Antimicrob Agents Chemother 46:1723-1727.

Williams DW, Wilson MJ, Lewis MA, and Potts AJ. 1995. Identification of Candida species by PCR and restriction fragment length polymorphism analysis of intergenic spacer regions of ribosomal DNA. J Clin Microbiol 33:2476-2479.

CHAPTER 6

CONCLUSIONS

Eukaryotic cell plasma membranes perform a number of diverse functions that regulate cell survival and function in response to environmental signals. The diverse role of plasma membranes in cellular function is especially apparent in human neutrophils. For example, neutrophils are actively maintained in a quiescent cellular state during blood circulation by soluble proteins present in plasma that prevent granule release in the absence of proinflammatory signals (Tschesche et al., 1994;Balke et al., 1995). In the presence of proinflammatory mediators, blood-neutrophils undergo a progression of rapid, transient changes in morphology, intercellular adhesion and migratory capacity that enables their targeted extravasation into infected tissues and subsequent microbicidal function (Perper et al., 1974;Sengelov et al., 1993a;Seely et al., 2003). A central question to understanding how neutrophils distinguish between negative and positive external signals and thus commit to processes of cellular activation concerns the role of plasma membranes in organizing and regulating signal transmission to the cell interior.

This work has focused, in part, on the examination of structural changes in plasma membranes that occur during initial stages of neutrophil activation. The work presented in Chapter 2 described the development of a model system that correlated the initial activation of human neutrophils with the selective exocytosis of secretory vesicles. An important aspect of this work investigated the physical and biochemical properties of secretory vesicle integration at the cell surface.

Previous studies employing electron microscopy coupled with cytochemistry to visualize the exocytosis of individual secretory vesicles within activated single cells found evidence of an unexpected complexity of vesicle interactions leading to their compound exocytosis and incomplete integration into existing plasma membrane structure (Kobayashi and Robinson, 1991;Kobayashi et al., 1998a;Kobayashi et al., 1998b;Robinson et al., 1999). These findings suggested a basis domain formation within activated plasma membranes that entailed the differential integration of surface-upregulated secretory vesicle membranes. This possibility was eliminated in the activated neutrophil population examined in Chapter 2 by demonstrating the homogeneous surface intergration of secretory vesicle-specific markers in activated neutrophils following sucrose density sedimentation of N_2 cavitates. Further evidence of the complete integration of secretory vesicles within existing structure of activated plasma membranes was demonstrated by a shift in peak density of the plasma membrane compartment from 29% sucrose in nonactivated neutrophils to a sucrose density (31%) in activated neutrophils intermediate between plasma membranes (29% sucrose) and intact secretory vesicles (34% sucrose) prior to their upregulation.

The work described in Chapter 2 also demonstrates the marked sensitivity of secretory vesicles in response to blood-neutrophil purification and the differential impact dextran-based and gelatin-based protocols on the cellular physiology of isolated populations. These protocols differed primarily on the basis of their effect on the stability of the secretory vesicle fraction, which was largely preserved in dextran-prepared neutrophils but absent from gelatin-prepared neutrophils. Further insight into the

exquisite sensitivity of the secretory fraction to different conditions of blood-neutrophil isolation was obtained from further work with the gelatin procedure. Introducing minor variations in the gelatin protocol resulted in either the complete loss or in the variable retention of secretory vesicles from isolated neutrophils. Prolonging incubation time at 37ºC during initial erythrocyte sedimentation led to no measurable variation in secretory vesicle upregulation. This was unexpected given that exposure to elevated temperatures in the absence of agonists results in spontaneous loss of secretory vesicles within *in vitro* isolated neutrophils. Instead, the factors contributing most to the stability of secretory vesicles was associated with intermediate steps between gelatin incubation and red cell lysis and related directly to the re-use of sedimentation tubes and pipettes for sedimentation and cellular resuspension, respectively. The repeated use of plasticware was associated with the variable retention of secretory vesicles in isolated neutrophils. In contrast, the use of sterile plasticware during each isolation step resulted in the complete loss of secretory vesicles. One possible explanation for the cellular impact of this minor variation pertains to the regulatory interaction of certain plasma proteins with circulating neutrophils *in vivo* (Tschesche et al., 1994;Balke et al., 1995;Haag-Weber and Horl, 1995;Horl, 2002). The presence of residual plasma proteins in previously used pipettes and sedimentation tubes could therefore modulate the activation state of neutrophils and inhibit secretory vesicle upregulation, thus leading to the variable retention of this fraction in isolated neutrophils. This finding was exploited to obtain neutrophil populations devoid of secretory vesicles. The established association of secretory vesicle

loss with neutrophil activation thus allowed the classification of gelatin-prepared neutrophils as activated and dextran-prepared neutrophils nonactivated on the basis of relative differences in secretory content (Borregaard et al., 1990;Borregaard et al., 1992;Sengelov et al., 1993b;Borregaard et al., 1994).

The work presented in Chapter 3 uses the model system developed in Chapter 2 to investigate the impact of cellular activation processes on the structural organization of plasma membranes during the initial stages of neutrophil activation. Plasma membrane structure can be simplified into three primary components, including an outer glycocalyx and an underlying cortex that are separated from each other by an intervening membrane bilayer (Mouritsen and Bloom, 1993). Of these, the most significant components in terms of organizing receptor-mediated signaling pathways and associated functional responses is the cortical cytoskeleton. Although the membrane bilayer is additionally implicated, direct evidence of such functional modulation by membrane lipid domains is lacking in human neutrophil studies. This is partly due to their elusive, apparently unstable construction in mammalian cell plasma membranes (Munro, 2003;Chamberlain, 2004;Lommerse et al., 2004;London, 2005). Conversely, the membrane cortex is, by definition, a structurally stable component of plasma membranes that can be readily measured. The work described in Chapter 3 tracked activation-induced changes in cortical structure using the primary structural components, actin and fodrin, as marker proteins. The magnitude of cortical change that accompanied cellular activation was indicated by relative differences in the subcellular fractionation pattern of actin and fodrin in nonactivated versus activated neutrophils. These studies indicated that cortical

remodeling during the early stages of human neutrophil activation is accomplished by the reorganization of primary cortical proteins from a loose association with nonactivated plasma membranes to a tight interaction with activated membranes. This conclusion is based on the respective susceptibility and resistance of cortical actin and fodrin to the sheer stress generated during the N_2 cavitation of nonactivated and activated plasma membranes (Lorenz and Willard, 1978;Schade and Levine, 2002). Accordingly, most of cortical actin and fodrin in nonactivated neutrophils was found in the cytosolic compartment following sucrose density gradient sedimentation, whereas these cortical components largely fractionated within gradient regions of plasma membrane enrichment in activated neutrophils. These findings were verified by compositional analysis of *in vitro* isolated membrane skeletons.

The specialization of actin and fodrin remodeling in activated plasma membranes was further indicated by their overlapping sedimentation patterns in sucrose density gradients. In particular, plasma membrane-associated actin and fodrin activity was shifted relative to the peak plasma membrane activity into denser regions of plasma membrane enrichment. This indicated that cortical proteins were likely associated with activated membranes in a nonuniform or clustered distribution, with the net result of making some regions of plasma membrane structure higher in relative density than other, neighboring regions (Figure 5, Chapter 3).

The possible functional significance associated with cortical remodeling in activated neutrophils was indicated by cosedimentation patterns observed for components of either the actin or fodrin cytoskeletons. For example, fodrin integration within

activated plasma membranes was accompanied by cell surface translocation of the fodrin anchoring protein, CD45. The peak subcellular distribution of CD45 localized to ~ 34% sucrose and thus significantly overlapped fodrin distribution. In addition, the cosedimentation actin with ezrin and the actin-anchoring protein, CD43, in sucrose density gradients further indicated that the functional competence of actin-enriched domains detected in activated plasma membranes.

The data presented in Chapter 3 indicate that the transition of neutrophils from nonactivated to activated cellular states is accompanied by a selective, extensive remodeling of the cell cortex, with the formation of distinct structural domains. Domain formation could be further correlated with secretory vesicle exocytosis and functional priming of activated neutrophils. The finding that activated populations were also primed for superoxide generation upon agonist stimulation and enhanced cellular adhesion in the absence of agonist was anticipated due to the absence of secretory vesicles in this population. The cortical rearrangements observed in activated neutrophils could thus be correlated with functional priming and selective secretory vesicle loss in activated neutrophils. Collectively, these data suggest that changes in plasma membrane structure accompany changes in cellular function and indicate the importance of cortical remodeling to the organization of signal transduction in human neutrophils.

Data from the study presented in Chapter 4 indicate a novel role of activated neutrophil plasma membranes in the function of the cathelicidin, hCap. The selective interaction of native hCap with activated, as opposed to nonactivated, plasma membranes suggests several parallels to the function of typical membrane domains. For example, by

binding to native hCap, the extracellular surface activated plasma membranes function as a platform capable of contributing to the spatial localization of hCap activation. Several observations point to the physiological importance of hCap-membrane interactions *in vivo*. First, hCap is a soluble protein capable of diffusing away from sites of their initial exocytosis. This suggests that the selective secretion of hCap at inflammatory sites could lead to its activation in noninflamed tissues and tissue-injury. Secondly, activated neutrophils undergo a limited degranulation during diapedesis and chemotaxis (Sengelov et al., 1993a). Thus, the capacity of plasma membranes to bind native hCap could act as a recovery mechanism to minimize the spread of potentially damaging inflammatory molecules to noninflamed sites. In addition, hCap-membrane interaction would insure the inclusion of hCap within phagosomes and the increased efficacy of microbial killing.

Although the antibiotic peptide component of hCap structure, LL-37, has been previously shown to bind mammalian cell plasma membranes (Malm et al., 2000), the study presented in Chapter 4 is the first to demonstrate the membrane binding capacity of the native molecule. Importantly, membrane-attachment of the antibiotic peptide leads to cell lysis. Consequently, LL-37 binding of mammalian cell plasma membranes could be attributed to the nonspecific nature of the innate arm of immune defense. Because the binding the native hCap to mammalian cell membranes does not result in cellular damage, an alternative functional basis for this interaction is likely, possibility relating to the spatial regulation of hCap activation.

The properties of hCap-membrane associations encompass a wide range of binding affinities from very weak interactions that can be eliminated by washing

membranes in saline to high affinity salt-resistant associations. The latter salt-resistant associations additionally co-partition with transmembrane proteins into a detergent-rich fraction following phase separation of cellular lysates using Triton X-114 (Borregaard and Tauber, 1984), consistent with the hydrophobic insertion of native hCap as a basis for membrane association. Significantly, in this respect, such high affinity hCap-membrane associations are sensitive 10 mM NaOH, which suggests basis for membrane attachment that is essentially nonhydrophobic. Collectively, these data suggest the most likely basis of hCap association with activated membranes involves a high affinity binding to one or more unidentified transmembrane protein receptors, although further research is needed to confirm this possibility.

The study discussed in Chapter 5 described the isolation and identification of mutant strains of yeast opportunist, *Candida glabrata*. These bile-dependent phenotypes presumably resulted from the *in vivo* persistence and eventual adaptation of organisms to anatomical sites rich in bile body fluids. These genetically modified organisms could no longer survive on conventional nutrient media used to selectively culture Candida glabrata but were still capable of causing disease. Because of their modified nutritional requirements, bile-dependent strains could not be detected in conventional microbiological tests designed to select for fungal growth. Thus, patients infected with bile-dependent organisms were misdiagnosed and/or ineffectively treated. The significance of this study is that it demonstrates how spontaneous genetic alterations can affect cellular metabolism and nutrient requirements in order to aid cellular survival in lipid-rich environments.

As cholesterol is the primary component of bile, one implication is that bile-dependent organisms undergo selective alterations in plasma membrane lipid biochemistry and/or lipid metabolism. Exposure of eukaryotic plasma membranes to markedly enhanced cholesterol concentrations alters the morphology and fluidity of membrane bilayers. Cholesterol-enriched membranes are thus compromised in structural integrity and functionally defective. Therefore the survival of bile-dependent organisms in cholesterol-enriched environments likely involves genetic alterations that directly concern plasma membrane structure and function. Other changes affecting cellular metabolism and signal transduction are also likely. Taken together, therefore, bile-dependent strains of Candida glabrata suggest the relative adaptability of plasma membrane structure and general cellular metabolic requirements in aiding cell survival in otherwise lethal conditions of markedly enhanced cholesterol-enrichment.

LITERATURE CITED

Balke N, Holtkamp U, Horl WH, and Tschesche H. 1995. Inhibition of degranulation of human polymorphonuclear leukocytes by complement factor D. FEBS Lett 371:300-302.

Borregaard N, Christensen L, Bejerrum OW, Birgens HS, and Clemmensen I. 1990. Identification of a highly mobilizable subset of human neutrophil intracellular vesicles that contains tetranectin and latent alkaline phosphatase. J Clin Invest 85:408-416.

Borregaard N, Kjeldsen L, Rygaard K, Bastholm L, Nielsen MH, Sengelov H, Bjerrum OW, and Johnsen AH. 1992. Stimulus-dependent secretion of plasma proteins from human neutrophils. J Clin Invest 90:86-96.

Borregaard N, Kjeldsen L, Sengelov H, Diamond MS, Springer TA, Anderson HC, Kishimoto TK, and Bainton DF. 1994. Changes in subcellular localization and surface expression of L-selectin, alkaline phosphatase, and Mac-1 in human neutrophils during stimulation with inflammatory mediators. J Leukoc Biol 56:80-87.

Borregaard N and Tauber AI. 1984. Subcellular localization of the human neutrophil NADPH oxidase. b-Cytochrome and associated flavoprotein. J Biol Chem 259:47-52.

Chamberlain LH. 2004. Detergents as tools for the purification and classification of lipid rafts. FEBS Lett 559:1-5.

Haag-Weber M and Horl WH. 1995. The immune system in uremia and during its treatment. New Horiz 3:669-679.

Horl WH. 2002. Hemodialysis membranes: interleukins, biocompatibility, and middle molecules. J Am Soc Nephrol 13 Suppl 1:S62-S71.

Kobayashi T and Robinson JM. 1991. A novel intracellular compartment with unusual secretory properties in human neutrophils. J Cell Biol 113:743-756.

Kobayashi T, Robinson JM, and Seguchi H. 1998a. Identification of intracellular sites of superoxide production in stimulated neutrophils. J Cell Sci 111 (Pt 1):81-91.

Kobayashi T, Zinchuk VS, Okada T, del Saz EG, and Seguchi H. 1998b. Intracellular dynamics of alkaline phosphatase-containing granules in electropermeabilized human neutrophils. Histochem Cell Biol 110:395-406.

Lommerse PH, Spaink HP, and Schmidt T. 2004. In vivo plasma membrane organization: results of biophysical approaches. Biochim Biophys Acta 1664:119-131.

London E. 2005. How principles of domain formation in model membranes may explain ambiguities concerning lipid raft formation in cells. Biochim Biophys Acta 1746:203-220.

Lorenz T and Willard M. 1978. Subcellular fractionation of intra-axonally transport polypeptides in the rabbit visual system. Proc Natl Acad Sci U S A 75:505-509.

Malm J, Sorensen O, Persson T, Frohm-Nilsson M, Johansson B, Bjartell A, Lilja H, Stahle-Backdahl M, Borregaard N, and Egesten A. 2000. The human cationic antimicrobial protein (hCAP-18) is expressed in the epithelium of human epididymis, is present in seminal plasma at high concentrations, and is attached to spermatozoa. Infect Immun 68:4297-4302.

Mouritsen OG and Bloom M. 1993. Models of lipid-protein interactions in membranes. Annu Rev Biophys Biomol Struct 22:145-171.

Munro S. 2003. Lipid rafts: elusive or illusive? Cell 115:377-388.

Perper RJ, Sanda M, Chinea G, and Oronsky AL. 1974. Leukocyte chemotaxis in vivo. II. Analysis of the selective inhibition of neutrophil or mononuclear cell accumulation. J Lab Clin Med 84:394-406.

Robinson JM, Kobayashi T, Seguchi H, and Takizawa T. 1999. Evaluation of neutrophil structure and function by electron microscopy: cytochemical studies. J Immunol Methods 232:169-178.

Schade AE and Levine AD. 2002. Lipid raft heterogeneity in human peripheral blood T lymphoblasts: a mechanism for regulating the initiation of TCR signal transduction. J Immunol 168:2233-2239.

Seely AJ, Pascual JL, and Christou NV. 2003. Science review: Cell membrane expression (connectivity) regulates neutrophil delivery, function and clearance. Crit Care 7:291-307.

Sengelov H, Kjeldsen L, and Borregaard N. 1993a. Control of exocytosis in early neutrophil activation. J Immunol 150:1535-1543.

Sengelov H, Kjeldsen L, Diamond MS, Springer TA, and Borregaard N. 1993b. Subcellular localization and dynamics of Mac-1 (alpha m beta 2) in human neutrophils. J Clin Invest 92:1467-1476.

Tschesche H, Kopp C, Horl WH, and Hempelmann U. 1994. Inhibition of degranulation of polymorphonuclear leukocytes by angiogenin and its tryptic fragment. J Biol Chem 269:30274-30280.