Hydrodynamic analysis of human neutrophil N-formyl chemotactic receptor-G protein interactions : mapping of interfacial domains with receptor-mimetic peptides

by Rajani Kanth Bommakanti

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry
Montana State University

Abstract:
The neutrophil's ability to seek and destroy invading pathogens is mediated by specific cell surface receptors. Bacterial protein synthesis initiation fragments, which contain a formylated methionine at their N-terminus, act as chemoattractants to neutrophils. Stereospecific binding of these peptides (agonists) to the N-formyl peptide chemoattractant receptor (FPR) leads to activation of various cellular functions which ultimately result in microbicidal activity. Specific interaction between agonist-occupied receptor and an intracellular guanine nucleotide binding protein (G protein) initiates a complex signal transduction pathway. These types of pathways have attracted a great deal of attention because of the many physiological and pharmacological events regulated by G proteins. There have previously been no studies to show direct interactions between a G protein-coupled receptor and G protein. Using a reconstitution assay involving hydrodynamic analysis of protein complexes, a direct interaction between agonist-occupied FPR and G protein from two different sources has been demonstrated. Uncomplexed FPR and FPR complexed with Gi2 exhibit two different molecular size forms which can be resolved by sedimentation analysis in detergent-containing linear sucrose density gradients. The ability of detergent solubilized FPR to bind reversibly to purified G protein was characterized under a variety of biochemical conditions. The inability of ADP-ribosylated Gi to bind to FPR was also demonstrated for the first time. Results also indicate that the agonist-bound FPR activates exogenously added Gi, by receptor induced release of bound GDP from Giα.

Based on the primary structure of FPR and its homology to various G protein coupled receptors, a working model for the topography of FPR has been proposed. The structural determinants of FPR required for its physical interaction with Gi were investigated using site-specific synthetic FPR peptides. Peptides corresponding to putative intracellular FPR domains were tested for their ability to interfere with FPR-Gi association. These peptide competition experiments suggest that at least four distinct FPR domains are involved in coupling to Gi, including the putative first cytoplasmic loop and a region (CTE) on the carboxyl terminal tail. These regions on other structurally related receptors have not been implicated in G protein interaction. In conclusion, evidence has been presented to demonstrate for the first time that a guanine nucleotide sensitive physical complex forms between FPR and two related G proteins, allowing investigation of the structural basis of physiologically relevant molecular interactions using synthetic FPR peptides.

HYDRODYNAMIC ANALYSIS OF HUMAN NEUTROPHIL N-FORMYL CHEMOTACTIC RECEPTOR-G PROTEIN INTERACTIONS: MAPPING OF INTERFACIAL DOMAINS WITH RECEPTOR-MIMETIC PEPTIDES

by

Rajani Kanth Bommakanti

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

of

Doctor of Philosophy

in

Biochemistry

MONTANA STATE UNIVERSITY
Bozeman, Montana

January 1994

APPROVAL

of a thesis submitted by

Rajani Kanth Bommakanti

This thesis has been read by each member of the thesis 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 College of Graduate Studies.

Chair, Graduate Committee — Date 4/22/94

Approved for the Department of Chemistry and Biochemistry

Head, Department of Chemistry and Biochemistry — Date 4/22/94

Approved for the College of Graduate Studies

Graduate Dean — Date 5/11/94

STATEMENT OF PERMISSION TO USE

In presenting this thesis 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 thesis 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 thesis should be referred to University Microfilms International, 300 North Zeeb Road, Ann Arbor, Michigan, 48106, to whom I have granted "the exclusive right to reproduce and distribute my dissertation for sale in and from microform or electronic format, along with the right to reproduce and distribute my abstract in any form in whole or in part."

Signature Rajani Bommakanti Date April 22, 1994

This thesis is dedicated to my beloved grandmother *Srimathi* Rapaka Varalakshmi who passed away on the 6th of December 1993. I am deeply indebted to her for her solicitude and interest in me. I express my heartfelt gratitude to her for sharing with me her insights into Hindu spirituality, which was and will always be my life's guiding principle.

VITA

Rajani Kanth Bommakanti was born on the day of Bhishmekadasi in the year Subhakruthu of the Hindu calendar (equivalent to February 3, 1963) to Suvani and Rama Rao Bommakanti in the east central Indian city of Vijayawada. After initial schooling in Kolar Gold Fields, Rajamundry and Hyderabad, Rajani attended Osmania University and graduated with a bachelors degree in Geology (Chemistry and Physics as ancillaries) and scored the top rank in the college. Later, Rajani attended the Indian Institute of Technology, New Delhi, (IIT Delhi) where he obtained a masters degree in organic chemistry. Rajani continued his post-graduate work at Montana State University and joined the research laboratory of Dr. Al Jesaitis in summer 1989.

Awards:

Graduate Achievement Award, MSU, April 1994
AFCR Trainee Investigator Award 1992 and 1993
Henry Christian Research Excellence Award 1991 and 1992 (AFCR)
University Grants Commission, Junior Research Fellowship, India, 1987
Graduate M.Tech. (GATE) Fellowship, India, 1987

Publications:

Bommakanti, R.K., et al., *J.Biol.Chem.(1992) 267*: 7576-7581.
Bommakanti, R.K., et al., *J. Leukocyte Biology* (1993) 54:572-577
Jesaitis, A.J., Erickson, R.W., Klotz, K.-N., **Bommakanti, R.K.**, Siemsen, D.W. *J. Immunology (1993)* 151: 5653-5665.

Manuscripts Submitted:

Bommakanti, R.K., Dratz, E.A., and Jesaitis, A.J., Mapping Of G Protein Binding Sites on Human Neutrophil N-formyl Peptide Chemoattractant Receptor: Use of Synthetic Receptor-Mimetic Peptides in a Physical Reconstitution Assay.

Bommakanti, R.K., Siemsen, D.W., Dratz, E.A., and Jesaitis, A.J., Characterization of Octyl glucoside Solubilized Neutrophil N-formyl Peptide Chemoattractant Receptor and its Complex with Gi2 Protein by Velocity Sedimentation.

ACKNOWLEDGEMENTS

I sincerely thank Dr. Al Jesiatis for introducing me to the spectacular areas of receptor biochemistry, for being my research advisor, and for showing me unique ways to approach science. It was a great privilege to experience the breadth of his intellect and seemingly limitless energies. I am grateful to Drs. Mark Quinn, Edward Dratz, Sam Helgerson, Jean Starkey, Sam Rogers, and Charly Klotz for providing capable guidance and encouragement. I am extremely grateful to Dr. Edwin Abbott for being a great friend and for introducing me to Montana's "Himalayas" and Dr. Jesaitis.

Mr. Dan Siemsen and Dr. Quinn were valued coworkers and were very helpful, especially when I started my "bench work"-- and for this I am most appreciative. I thank our current and former group members, Lisa Mullen, Stuart Sheets, Goutam Mukherjee, Jeannie Gripentrog, Linda Loetterle, Greg Gehlen, and Kathy Sandoval as well as Drs. Arunas Gineitis, Heini Miettinen and John Linner for putting up with me and my culinary experiments. My thanks to all the staff, faculty, and graduate students in the Cooley labs and Gaines Hall for their friendship. My special admiration to Earl Adams for all the laughter, "F-16 dog fights" on the work stations, and the saporific brussels sprouts recipes.

I derived strength and motivation while under the tutelage of many current and former teachers, especially Ms. Imamunnesa Begum (Biology, High School), Drs. G.N.Rao, I. Habeebulla, and V. Manaiah (Chemistry, College), and Dr. Elizabeth Viau (English/Philosophy, Graduate School).

I have immensely enjoyed the warm company and friendship of the following friends and their families: Dr. and Mrs. Edward Anacker, Dr. and Mrs. Frank Johnson, Dr. Cliff Bond, Dr. Margaretha Wessel, Dr. Erik Van Kuijk, Paul and Karen McMullin in Ennis, MT., Dorothy Miller, Susie and Joel Leadbetter, Pamela Mead, Valarie Simenson, and Ilka and Tim Freidrichson in Berlin. I must thank all my friends from India, Sri Lanka, Nepal, Bangladesh and Pakistan for their help in organizing festivals, all the plethora of dinner invitations/hospitality, chess games and humor.

Finally, I must look to my beginnings - my family. I cannot express enough my deep appreciation and respect for my mother and father, Suvani and Rama Rao, for making me who I am today, but I am glad they are my parents, just as I am for my sister, Aruna, for her affection and my brother, Gopi for being the best critic one could ever have. Our family prays for the soul of my cherubic and beloved sister Madhavi Latha.

TABLE OF CONTENTS

TABLE OF CONTENTS--Continued

TABLE OF CONTENTS--Continued

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES--Continued

LIST OF FIGURES--Continued

ABSTRACT

The neutrophil's ability to seek and destroy invading pathogens is mediated by specific cell surface receptors. Bacterial protein synthesis initiation fragments, which contain a formylated methionine at their N-terminus, act as chemoattractants to neutrophils. Stereospecific binding of these peptides (agonists) to the N-formyl peptide chemoattractant receptor (FPR) leads to activation of various cellular functions which ultimately result in microbicidal activity. Specific interaction between agonist-occupied receptor and an intracellular guanine nucleotide binding protein (G protein) initiates a complex signal transduction pathway. These types of pathways have attracted a great deal of attention because of the many physiological and pharmacological events regulated by G proteins. There have previously been no studies to show direct interactions between a G protein-coupled receptor and G protein. Using a reconstitution assay involving hydrodynamic analysis of protein complexes, a direct interaction between agonist-occupied FPR and G protein from two different sources has been demonstrated. Uncomplexed FPR and FPR complexed with Gi2 exhibit two different molecular size forms which can be resolved by sedimentation analysis in detergent-containing linear sucrose density gradients. The ability of detergent solubilized FPR to bind reversibly to purified G protein was characterized under a variety of biochemical conditions. The inability of ADP-ribosylated Gi to bind to FPR was also demonstrated for the first time. Results also indicate that the agonist-bound FPR activates exogenously added Gi, by receptor induced release of bound GDP from Giα.

Based on the primary structure of FPR and its homology to various G protein coupled receptors, a working model for the topography of FPR has been proposed. The structural determinants of FPR required for its physical interaction with Gi were investigated using site-specific synthetic FPR peptides. Peptides corresponding to putative intracellular FPR domains were tested for their ability to interfere with FPR-Gi association. These peptide competition experiments suggest that at least four distinct FPR domains are involved in coupling to Gi, including the putative first cytoplasmic loop and a region (CTE) on the carboxyl terminal tail. These regions on other structurally related receptors have not been implicated in G protein interaction. In conclusion, evidence has been presented to demonstrate for the first time that a guanine nucleotide sensitive physical complex forms between FPR and two related G proteins, allowing investigation of the structural basis of physiologically relevant molecular interactions using synthetic FPR peptides.

CHAPTER 1

INTRODUCTION

General

Neutrophils typically constitute about 65% of all white blood cells in humans and are considered as the first line of defense against invading pathogens. Owing to the lobed nuclear morphology, neutrophils are also called polymorphonuclear leukocytes (PMN's). Stimulated neutrophils carry out a series of biological and biochemical processes which ultimately lead to the killing of microorganisms. Specific cell surface receptors on neutrophils act as molecular antennas and help the cell "home in" to sites of infection so that they can suitably unleash their microbicidal potential. Critical to the activation of neutrophil is the ability of the cell surface receptors to recognize a heterogenous group of agonists called chemoattractants. Peptide chemoattractants include certain formylated peptides; a complement protein fragment, C5a; and a lymphokine, interleukin 8, while lipid chemoattractants include leukotriene B4 (LTB4) and platelet-activating factor (PAF). Receptor proteins for each of these chemoattractant agonists have been cloned and sequenced (for a recent review see [1]).

Formylated peptide mediated events are among the better understood eukaryotic chemotactic signal transduction pathways. N-formyl methionyl modification of proteins occurs during the initiation of bacterial protein synthesis. Bacteria release N-formyl methionyl peptides at the site of infection, and their subsequent entry into the circulatory system activates neutrophils and triggers their chemotaxis (directed migration) to the site of infection. Thus, N-formyl peptide chemoattractant receptors (FPR) permit neutrophils to play a role in defense against bacterial infections.

The binding of formyl peptides to FPR is stereospecific and causes activation two major groups of responses. Responses primarily related to chemotaxis such as morphological polarization, cytoskeletal rearrangement, stimulated and directed locomotion occur at low chemoattractant doses (< 2 nM), and microbicidal or secretory functions such as lysosomal enzyme secretion and activation of the respiratory burst require at least 10 fold higher concentrations of the same agonists [2]. Evidence that the two basic functions are discrete comes from studies involving pharmacological agents which can modify receptors' affinity for agonist and selectively enhance or depress one or the other function [3]. The responses described help protect the host against infection, inappropriate stimulation of this sensory system is also implicated in pathogenesis of certain diseases.

Eukaryotic mitochondria also initiate protein synthesis with N-formyl methionine. Mitochondrial DNA programs the synthesis of a set of hydrophobic

proteins, found in the respiratory complexes in mitochondrial membranes. N-formyl peptides, released by degeneration of the body's own mitochondria at sites of tissue damage, may thus promote accumulation of neutrophils at these sites [4]. The mitochondrial respiratory chain normally spills some free radicals from the four one electron reduction steps of molecular oxygen to water. Such free radicals are thought to be toxic and additional free radicals could be generated by neutrophils in response to mitochondrial N-formyl peptides as well as to bacterial sources. An over abundance of radicals may excessively damage the host tissue [5]. Moreover, inappropriately activated neutrophils at sites of inadvertent tissue damage could lead to potentially deleterious effects. Supporting this argument is the neutrophils destructive potential which has been implicated in a number of disease states including arthritis [6], ischemic heart disease [7], periodontitis [8], adult respiratory distress syndrome (ARDS) [9] and certain types of inflammatory cancers [10]. Although the role of FPR in these processes has not been established, investigation of neutrophil activation pathways involving FPR may be important in the understanding and controlling inappropriate neutrophil activation and moderation or possibly even cures of such disease states.

Chemoattractants

Formylated peptides were found to be neutrophil chemoattractants in 1975 by Schiffmann and colleagues [11]. Showell et al, [12] initiated studies of

structure-activity relationships of N-formyl peptides. N-formyl peptide research spanning nearly two decades, resulted in the emergence of N-formyl-L-methionyl-L-leucyl-L-phenylalanine (popularly known as f-Met-Leu-Phe, fMLP or fMLF) as the prototypic chemotactic tripeptide. Excellent reviews of the structure function relationships were published by Showell et al [12], Freer et al [13], and Dentino et al [14]. Briefly, 1) The N-formyl-Met at position 1 and the Phe at position 3 are crucial for optimal activity of the peptide in activating neutrophils, whereas substitutions of the Leu at position 2 with bulky, hydrophobic sidechains are well tolerated [13]. 2) Using two f-Met-Leu-Phe conformationally "locked" analogues (one containing an extended beta conformation and other with a constrained folded conformation) Dentino et al [14] concluded that a) alterations in the backbone conformation at the tripeptide level may not significantly alter the side chain topography and/or accessibility of key functional groups important for interaction with the receptor, and b) FPR prefers ligands with extended back bone when they contain four or more amino acids.

Isolation and purification of N-formyl peptides from Escherichia Coli [15] and Streptococcus Sanguis [16] was also achieved. Demonstration that f-Met-Leu-Phe purified from bacteria are pan-activators (activation of various neutrophil events) of neutrophils implicates them (neutrophils) as host-defense mediators.

Information Transfer Across Cell Membranes

One of the ubiquitous features of living cells is the ability to communicate with the extracellular milieu. Though certainly not limited to transmembrane signalling, the phrase "signal transduction" is widely used to denote the transfer of biochemical information across the cell membrane. The plasma membrane is selectively permeable to molecules that are lipid soluble, but effective stimuli often include water soluble molecules in the extracellular fluids that do not penetrate cell membranes. Most water soluble molecules don't cross the membrane unless there is a specific channel. The transfer of information from the aqueous extracellular side to the intracellular metabolic machinery is achieved by specialized proteins called receptors. These receptors are typically deeply embedded in and span the plasma membrane. Although proteins interact with membranes in different ways, specific hydrophobic stretches in proteins are thought to promote the association of proteins with the hydrophobic interface of membranes. Segments of transmembrane signalling receptors are exposed to the extracellular environment where they can discriminate signals while the cytoplasmic surfaces mediate activation of intracellular events, and lead to cellular responses. The predominant signalling mechanisms in mammalian cells involve membrane spanning receptors, which interact concertedly with intracellular proteins to amplify the signals in a number of ways including generation of secondary messenger molecules.

There are four main classes of membrane-anchored receptors: a) receptors

which are also enzymes (such as tyrosine protein-kinase or guanylate cyclase), b) receptor channels that shuttle hydrophilic molecules across the membrane also known as transporters or exchangers, c) receptors coupled to GTP binding proteins, and d) receptors with unknown transduction mechanisms. Sensory signal transduction in eukaryotes, as mediated by various receptor protein classes, has been recently reviewed by Van Haastert et al, [17].

Adrenergic receptors and rhodopsin are among the most studied of transmembrane receptors, which interact with small biogenic amines and light, respectively. These receptors are among the best known examples of a large family of receptors which interact with guanine nucleotide binding proteins (G proteins). Activation of the specific G protein, Gs, by adrenergic receptors causes the stimulation of the enzyme adenylyl cyclase, which increases the level of a second messenger, cyclic AMP (cAMP), inside the cell. Rhodopsin activation of a G protein called tranducin (or Gt) leads to stimulation of a cGMP-specific phosphodiesterase that reduces the level of cGMP in the cell. Two decades of research involving receptors and G proteins has recently been summarized by Lutz Birnbaumer [18].

FPR

The FPR can be identified by photoaffinity labeling with a derivative of N-formyl peptide f-Met-Leu-Phe-Lys coupled to a heterobifunctional radioiodinated

cross-linker, f-Met-Leu-Phe-Lys-Nε-(2-(p-azido[^{125}I]salicyl-amido)ethyl-1,3'-dithopropionyl). This specific FPR labeling reagent is abbreviated fMLFK-[^{125}I]ASD, and was also formerly referred to as FMLPL-[^{125}I]SASD) [19]. The affinity-labeled FPR from human neutrophils behaves as a species of approximately 63 kDa when analyzed by equilibrium sedimentation analysis in Triton X-100 [20]. Similarly, the FPR from differentiated HL60 cells elutes as a 66 kDa species upon gel filtration in sodium cholate [21]. In either studies the amount of detergent bound to FPR was not measured. However, these estimates are consistent with the apparent size of the photoaffinity labeled receptor on reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, where the liganded receptor migrates as a broad band between 50 and 70 Kd band [20,22,23] characteristic of heterogenously glycosylated proteins. The gene sequence for the protein corresponds to 350 amino acid residues (discussed in the next paragraph) and has a theoretical molecular weight of 38401. The molecular weight derived from gene sequence is consistent with the reported molecular weight of a deglycosylated FPR [24] on SDS-PAGE, but differs slightly from earlier deglycosylation studies [25-27], in which a sharp band around 34 kDa has been suggested to be FPR.

Complementary DNA (cDNA) sequences have been obtained for four leukocyte chemoattractant receptors (reviewed in [1]). Boulay and coworkers [24,28] constructed a cDNA library from human leukemic (HL60) cells, that could

express FPR when transfected into COS-7 cells (African monkey kidney cells transformed by SV40 virus). A cDNA clone was isolated that conferred to COS-7 cells the ability to bind N-formyl peptides and this approach led to the isolation, cloning and sequencing of the N-formyl peptide chemoattractant receptor (FPR) gene coding for a single 350 amino acid poly peptide chain. This confirmed earlier predictions regarding the presence of a specific cell surface receptor for formylated peptides [29,30]. Ye and coworkers [31] published a report in which they screened a rabbit neutrophil cDNA library with a cloned human FPR cDNA probe and cloned and sequenced rabbit FPR. Sequence comparison indicates rabbit FPR has 78% amino acid sequence identity to human FPR.

It may be significant for results to be presented (see Appendix) that Murphy and coworkers [32] expressed human FPR in Xenopus laevis oocytes. Murphy and coworkers [32] also reported that functional expression of FPR **required** the coexpression of a complementary human factor. The complementary human factor did **not** correspond to the alpha subunits of G proteins, and investigations are underway to characterize the unknown factor. Subsequently the same investigators have cloned and sequenced two structural homologues of FPR from differentiated HL60 cells [33]. The FPR2 homologue has 69% sequence identity with human FPR and behaves similar to normal FPR with respect to ligand binding. The other homologue, termed FPR like-1 (or FPRL1), while also sharing 69% sequence identity with human FPR, does not bind prototypic N-formyl peptides suggesting

the existence of an unidentified peptide that may recruit neutrophils to sites of infection and/or inflammation.

The relatively modest expression of FPR on neutrophils (50,000-100,000 copies per cell) has been an obstacle to carrying out in depth structural and functional characterization. Attempts are being made to over-express functional FPR in a cultured cells. Lala and coworkers [26] have isolated, cloned and expressed a non-glycosylated full-length human FPR in *E. coli*. The FPR expressed in E.coli exhibits radioligand binding, that were competitively inhibited by excess non-radioactive ligand, therefore the authors concluded that the glycosylation is not required for labeling or activity of the FPR. This report also represents the first prokaryotic expression of FPR.

Quehenberger et al [34], have expressed and characterized recombinant human FPR in baculovirus infected Sf9 insect cells. They [34] report a hundred fold reduced value for the binding affinity of fMLF to cells expressing FPR and ascribed this to the absence of pertussis toxin substrate (G proteins) and the consequent lack of pre-coupling to Gi protein in these cells. Ali and coworkers [35] expressed an N-terminal epitope-tagged functional FPR in rat basophilic leukemia (RBL-2H3) cells. The epitope tag consisted of a nine amino acid peptide insert (YPYDVPDYA) between the N-terminal methionine and the second residue of FPR. A monoclonal antibody (12CA5) selectively reacts with the epitope thus facilitating purification and further functional characterization of FPR. These

investigators report functional expression of FPR (in RBL-2H3 cells) did not require cotransfection of any complementary factor which might have been present in the trasfection host.

Structural Features of FPR

Hydropathy analysis of FPR's derived amino acid sequence and the presence of characteristic conserved amino acids [36-38] provides strong evidence that FPR belongs to a family of receptors called G protein-coupled receptors (GPCR) [39]. The most striking feature of GPCR is the presence of seven predominantly hydrophobic stretches which are thought to form membrane spanning α-helical domains. The putative transmembrane domains are thought to be connected by relatively hydrophilic polypeptide domains on the extracellular and the cytoplasmic side of these receptors. The seven transmembrane helix motif was first discovered by electron diffraction analysis of bacteriorhodopsin, a non-G protein-linked light-driven proton pump [40]. There is considerable circumstantial evidence that many of the salient features of the bacteriorhodopsin tertiary motif are present in rhodopsin [41] and this has been partially validated for rhodopsin [42] and less so for β2-adrenergic receptor (reviewed in [39]).

Based on sequence homology between FPR, rhodopsin, and other GPCR we have proposed a model for the transmembrane organization of FPR [43] (Figure 1). The model predicts three potential N-linked glycosylation sites on the extracellular

side, seven transmembrane spans, an amino terminus facing outside the cell and a COOH terminus on the cytoplasmic side with three cytoplasmic loops connecting the membrane helicies. A related but significantly different model has been proposed for human [24] and rabbit FPR [31]. The latter two models predict a relatively short putative third cytoplasmic loop compared to the first model described [43] and this difference is relevant to some of the data to be presented in chapter 5 and the Appendix.

Sequence alignment of FPR with various GPCR reveal residues that are conserved in virtually all of the receptors [36,37,38,44]. Highly conserved residues among various GPCR include, cysteines in each of the first two extracellular loops, an aspartate/glutamate-arginine diad (D(E)/R) near the cytoplasmic interface of the third transmembrane domain, several hydrophobic residues in the membrane spanning domains and proline residues in the transmembrane domains 4, 5, 6 and 7.

Comparison of the entire protein sequences reveal at most 20-25% amino acid identitity (entire protein compared). Such identities are modestly higher when FPR is compared with the sequences of other eukaryotic chemoattractant receptors and is highest (34%) against human complement derived C5a receptor. Comparison of the predicted individual cytoplasmic loops of FPR and other eukaryotic chemoattractant receptors (viz., C5a receptor, interleukin-8 receptor, platelet-activating factor receptor, and cyclic AMP receptor from Dictyostelium)

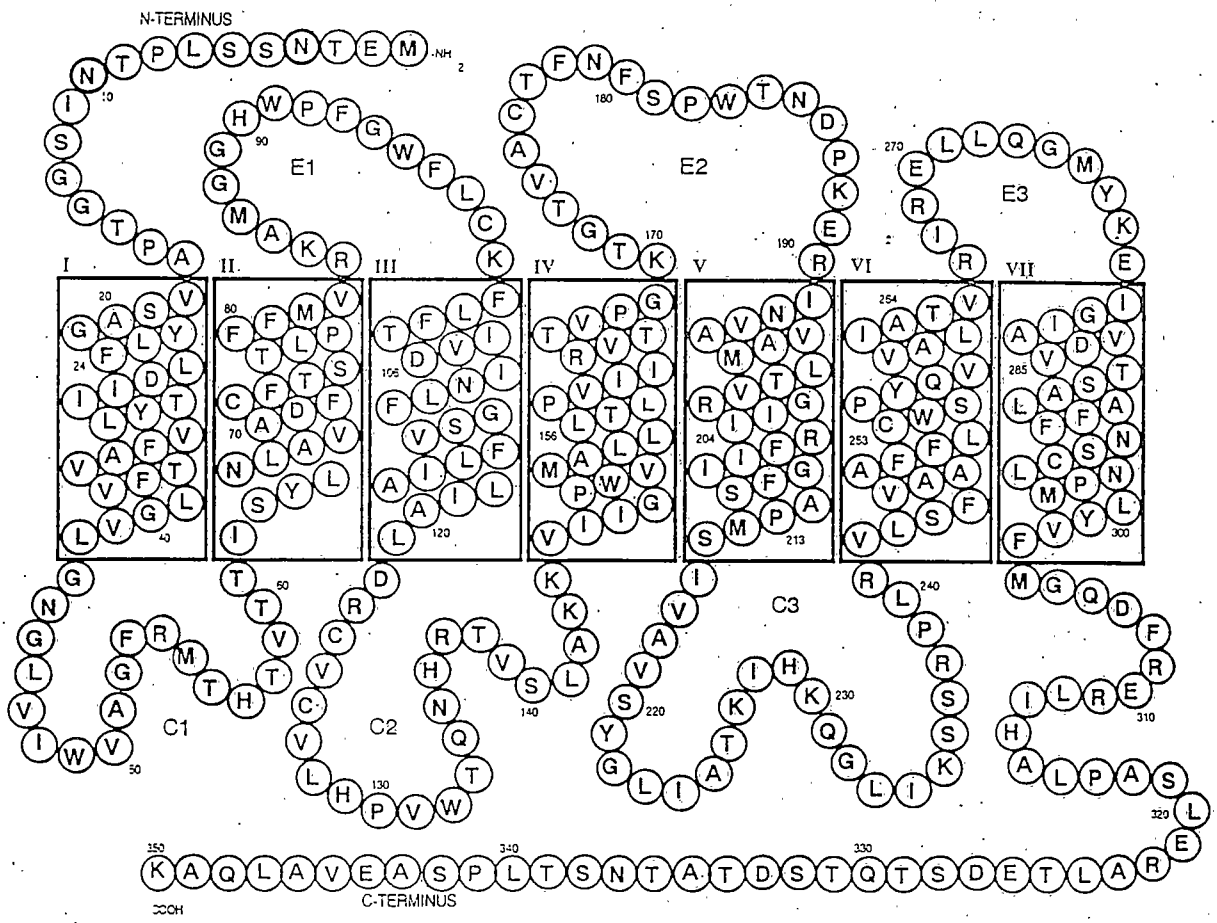

Figure 1: Schematic model of the FPR polypeptide chain in the neutrophil plasma membrane. I through VII represent the putative lipid bilayer spanning domains, which are connected by loops E1, E2, and E3 on the extracellular side and loops C1, C2, and C3 on the cytoplasmic side of the membrane. The asparagine residues at amino acid positions 4,10, and 179 are predicted to be three potential glycosylation sites on the receptor.

reveal sequence identities ranging from 13% (second cytoplasmic loops of FPR and cAMP receptor) to 42% (first cytoplasmic loops of FPR and C5a receptor). When comparisons in the loop regions were extended to other G protein-coupled receptor subfamilies, for example, the receptors for neurotransmitters, vision, odorants, the sequence identities ranged from 3% (the third cytoplasmic loops of FPR and rat substance P receptor) to 35% (second cytoplasmic loops of FPR and rat substance P receptor). Notable high identities includes, the first cytoplasmic loops of FPR and rat substance P receptor, and the second cytoplasmic loop of human adenosine A_2 receptor, with 53% sequence identity in each case. The identities of these loop regions are consistent with receptor-specific contacts with G protein(s) as well as other regulatory proteins [45-47] and highlight the pardigm that conserved regions may lead to conserved structure (bacteriorhodopsin and rhodopsin) and nonconserved regions may represent potential specific interactions sites.

Ligand Binding to FPR

Information regarding the residues which are thought to interact with the ligands is rapidly evolving. The presence of a ligand binding pocket for the chromophore retinal deep within the transmembrane α-helices (TMH) of rhodopsin was suggested by the initial model [41] and supported by cross-linking and fluorescent energy transfer studies [36]. 3D crystals have not been reported for any of the G protein-coupled receptors to date, but the ligand binding sites on

rhodopsin, and of the adrenergic receptors have been partly elucidated through biochemical and molecular biological approaches (reviewed in [48,49]). These studies suggest that the transmembrane domains form a hydrophilic pocket for ligand binding surrounded by hydrophobic residues.

FPR may differ from many other GPCR in the placement of its ligand binding domain. Fay and coworkers [50] investigated the ligand binding site on FPR using fluorescent N-formyl peptide derivatives and concluded that the ligand binding pocket a) can accommodate at least five but not more than six amino acids, b) contains at least two micro environments on the receptor, one hydrophobic and another capable of supporting protonation, both of which can affect ligand binding and c) the only histidine on the extracullular side at position 90 (first extracellular loop) may be involved in the protonation and stabilization of ligand binding. The authors also suggested that several regions on the second and third extracellular loops as well as the transmembrane domains could potentially contribute to a ligand binding pocket since they contain stretches of hydrophobic residues in close proximity of protonatable amino acids. Since the fluorescent ligand binding and dissociation to FPR exhibited a transition point near pH 7.5, the authors speculate that the histidine residue at position 90 in the first extracellular loop may be responsible for the observed pH dependent changes in ligand binding. Although FPR residue(s) that may be protonated are not known, the authors conclude that any of the numerous lysines, arginines, acidic amino acids are potential candidates.

Radel et al [51], reported that a synthetic peptide corresponding to the first putative extracellular loop (especially the amino terminus half) specifically inhibited fMLF ligand bindign to neutrophil membranes. Peptides from other extracellular domains did not have any effect, suggesting a role for the first extracellular loop in ligand binding. Perez et al [52], using chimeras of FPR and C5a receptors, concluded that all three extracellular loops contribute to the ligand binding pocket, and that the N-terminus forms a "lid" to the pocket. Quehenberger et al [53], constructed a series of chimeras of FPR and its homologue FPR2 (whose Kd for fMLF is 450 fold lower than that for FPR). They reported that replacement of the FPR domains, including the first and the third extracellular loops, resulted in 275 and 85-fold decrease in ligand binding affinity and concluded that the major determinants for ligand binding are located in the first extracellular loop and the adjacent second TMH domain. These results concur with results reported by Radel et al [51] and Fay et al [50] but not with that of Perez et al [52]. Using a nonglycosylated FPR expressed in E.coli, Lala et al [26], reported that the glycosylation of FPR does not play a role on ligand binding . The model we have proposed for FPR (Figure 1) meets several criteria that are shown to modulate ligand binding, as well as the location of amino acid residues that are speculated to be protonated. The identification of the region(s) involved in ligand binding would further test the validity and the assignment of the transmembrane domains and the extracellular loops of FPR.

G Proteins

G proteins that transduce signals from the class of GPCR membrane receptors to intracellular effectors are heterotrimeric and belong to a distinct family of proteins. Three subunits α,β and γ form a complex in a 1:1:1 ratio [55]. Gα binds guanine nucleotides and has intrinsic GTPase activity where the terminal phosphate on bound GTP is cleaved to form GDP. In its basal state Gα contains bound GDP and is tightly associated with the βγ subunits. In the absence of excited receptors the exchange of bound GDP for GTP is extremely slow under physiological conditions [54,55]. The primary role of an agonist occupied receptor is to stimulate GDP release and thereby catalyze the GDP-GTP exchange. The βγ subunit complex greatly stimulates interaction between Gα and the receptor, and the nucleotide exchange (reviewed in [56]). Moreover, there is now evidence that the βγ subunits also contribute to the specificity of receptor-G protein coupling [57]. Upon activation, *in vitro*, Gα-GTP dissociates from the βγ subunits (see Figure 2). Both species that form after receptor stimulation (Gα-GTP and βγ) are now known to activate various cellular effectors such as adenylyl cyclases, phospholipases, and ion channels etc., (reviewed in [58,59]).

To date about 20 different Gα subunits have been cloned and sequenced. The Gα's are divided into four subfamilies Gs, Gi, Gq and G12 based on sequence homology. Molecular weights of the Gα subunits range from 39-46 kDa. Amino acid identity among members of the same subfamily ranges from between 55 to

95%, whereas identity among members of different subfamily is less than 45% (reviewed in [58]). The α subunit is relatively hydrophilic and does not appear to associate with phospholipid vesicles unless βγ subunits are present [60]. Several of the Gα subunits are modified both co- and posttranslationally [57]. Cotranslational modification is irreversible, depends on protein synthesis and involves addition of a 14 carbon myristate group on the amino-terminal glycine residue. This myristoylation (not all Gα are myristoylated) may be important in the membrane attachment of Gα. Posttranslational modification may also involve covalent coupling of palmitate to cysteine through a thioester bond [61]. Unlike myristoylation, palmitoylation is reversible and independent of protein synthesis [61].

Many α subunits are substrates of bacterial toxins released by *Vibrio cholera* and/or *Bordetella pertussis* in which an ADP-ribose moiety is coupled to an arginine or a cystine residue respectively [62]. Modification of α subunits by cholera toxin constitutively activates these proteins (by inhibiting their GTPase activity) [58], whereas modification by pertussis toxin prevents receptor-mediated activation of G proteins [63]. Ribosylation requires the presence of the full heterotrimer [62]. Apart from their role in coupling receptors to effectors, trimeric G proteins are also implicated in golgi transport [64].

The βγ subunits are much more hydrophobic than the α subunit and have a strong tendency to attach to phospholipid bilayers, and with each other. The βγ have not been shown to dissociate under physiological conditions. Four β subunit sequences are known, each consisting of about 340 amino acids and range in molecular weight between 35 and 37 kDa [55]. The β subunits share more than 80% amino acid identity. The amino terminal region is predicted to form an amphipathic α-helix which has been proposed to participate in its interaction with both α and γ subunits in a three stranded coiled coil [65]. The remainder of the β subunit consists of seven repeating units, each about 43 amino acids long and their role in signal transduction is not known. Structural characterization and the newly identified functional roles of G protein βγ subunits in signal transduction have been recently reviewed in [66-68].

Currently, seven different Gγ subunits are known. They are about 75 amino acids long and are much more heterogeneous than β subunits with sequence identities less than 40%. The Gγ subunits also differ in their modification by prenyl groups: Gγ1 is farnesylated; whereas Gγ2 is geranyl geranylated [67]. With the known number of G protein subunits and their tissue distribution it appears that nearly 1000 possible oligomeric combinations are possible [58,69,70]. Such combinatorial power may be used to give cells the capacity to fine tune the magnitude and the nature of their response to extracellular signals.

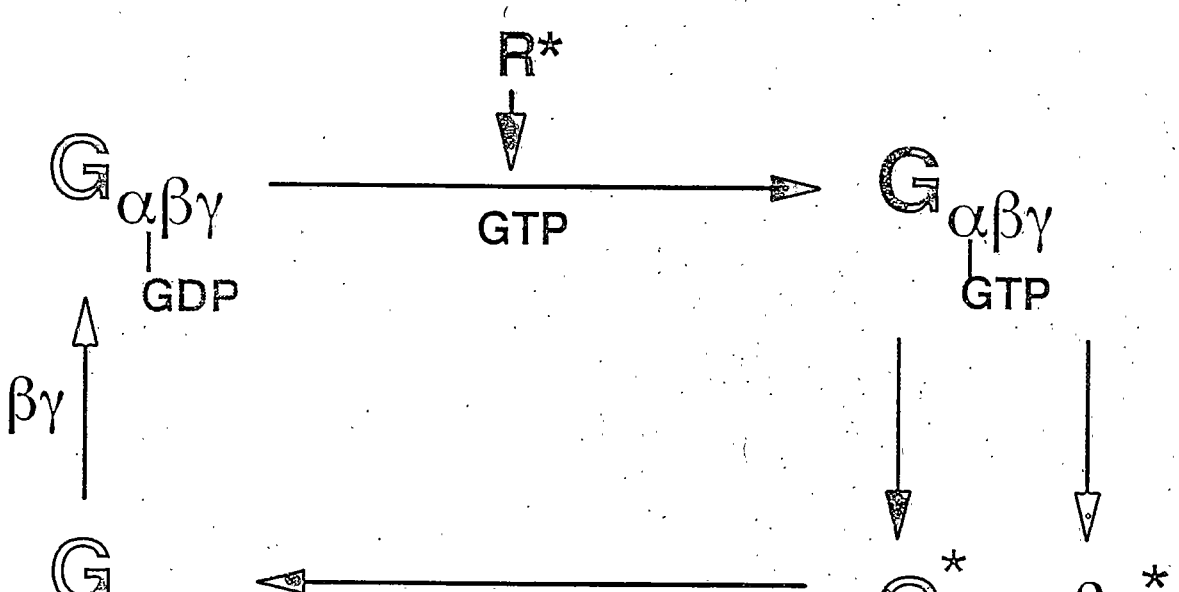

Figure 2: Diagram depicting the activation and deactivation cycle of G proteins. $G\alpha\beta\gamma$, R* represents the heterotrimeric G protein, and agonist occupied G protein-coupled receptor.

FPR-Gi Interactions

There is a substantial body of evidence suggesting that the chemoattractant receptors initiate signal transduction through interaction with G proteins [54,71,72]. Like hormone receptors, the N-formyl peptide chemoattractant receptor (FPR) in purified membranes [73] or in permeabilized neutrophils exists in a high affinity state for agonist that can be specifically converted to a low affinity state by guanosine 5'-O-(triphosphate) (GTP) which is a generally accepted manifestation of G protein coupling [54,56,74]. The non-hydrolyzable analogue of GTP (guanosine 5'-O-(γ-thiotriphosphate) or GTPγS) inhibits high affinity binding of fMLF to FPR with an IC_{50} of 20 nM. Studies also suggest that agonist mediated high affinity binding of ligand, GTPase activity, and activation of phospholipase C (PLC) can be restored to purified neutrophil membranes by exogenous rat or bovine brain Gi/Go mixtures [75,76]. Polakis and coworkers [21,77] have reported the copurification of FPR with a pertussis toxin substrate when detergent extracts of plasma membranes from differentiated HL60 cells were analyzed by gel filtration, ion exchange and affinity chromatography steps. Guanine nucleotides caused a reduction in ligand binding affinity to the isolated protein complex suggesting an association of FPR with G protein.

Jesaitis and coworkers [78], discovered that the octyl glucoside solubilized FPR from unstimulated cells exhibits two size forms with apparent sedimentation coefficients of 4 and 7S . Because the 7S form could be converted to the 4S form

by GTPγS with an IC_{50} value of 20 nM, the 7S form was hypothesized to represent a FPR-G protein complex. Another generally accepted criterion for receptor-G protein coupling, pertussis toxin-catalyzed ADP-ribosylation of a 40 kDa membrane substrate has also been demonstrated in neutrophils. Pertussis toxin also disrupts the functional coupling of FPR to neutrophil activation [79-81], and this process can be modulated by fMLF binding to the membranes. The molecular mechanism of the uncoupling however, remain unclear. In addition, chemotactic N-formyl peptides stimulate guanine nucleotide binding and pertussis toxin-sensitive GTPase activity in the neutrophil membranes [82,83].

Matsuoka et al [84], investigated the expression of Gα subunit genes in hematopoietic cells. Their studies indicate that Gi2 and Gi3 (40 and 41 kDa respectively) are expressed in high quantities in both myeloblasts and promyeloblasts, which are precursor cells for neutrophils. They also detected high levels of Gs (46 kDa), but virtually no detectable quantities of the Go, Gx and Gi1 subtypes. Moreover, partial sequence analysis of the major pertussis toxin substrate in neutrophils suggest that it belongs to Gi2 subtype (reviewed in [72]). Taken together these results indicate that FPR may be coupled to a Gi2 type protein.

It has been reported that activation of one FPR could lead to the activation of at least 20 G protein molecules in native HL60 cells (as assessed by FPR promoted GTPγS binding to Giα) [83]. Since Gi2 and Gi3 are expressed abundantly in neutrophils, they both might be involved in FPR mediated events

[85]. Given the potential for single receptor being able to activate more than one G protein (reviewed in [86]), it is important to delineate the G protein subtypes that interact with FPR.

Effector Regulation

The activation of intracellular effectors by FPR-G protein(s) interactions appears to be more complex than regulation of adenylyl cyclase by G proteins in hormone systems. FPR has the potential to activate at least three different phospholipases, A2, C, and D (reviewed in [87]) via G protein activation. It is not known, however, whether effector stimulation is via Gα or βγ or both, which subtypes of lipases are involved, or the mechanism of activation.

Cockcroft et al, [87] were the first to suggest that fMLF stimulated phospholipase D (PLD) in neutrophils. They measured the accumulation of labelled phosphatidic acid in neutrophils in response to fMLF. The same investigators previously demonstrated activation of inositide-specific phospholipase C in neutrophils stimulated with fMLF [88]. PLD cleaves membrane lipid phosphatidylcholine (PtdCho) into phosphatidic acid (PA) and choline, inositide-specific PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) to yield inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DG). IP3 releases Ca^{2+} from intracellular calcium stores and DG activates protein kinase C (PKC) [89].

PKC appears to have a central role in the receptor transduction pathway

leading to the generation of superoxide anion and other free radicals [90]. The initial product of the PLD pathway, PA, also appears to act as a second messenger by directly activating the NADPH oxidase responsible for generating superoxide anion [91]. Since PA and DG can be interconverted, it is not known which of these second messengers has the dominant role. Phospholipase A2 cleaves arachidonic acid from the sn-2 position of phosphatidylethanolamine, phosphatidylcholine or phosphatidylinositol. In neutrophils, arachidonic acid was implicated in exocytosis as well as activation and maintenance of the NADPH oxidase activity [87]. Release of arachidonic acid by fMLF is sensitive to inhibition by pertussis toxin. PLC activation is more sensitive to pertussis toxin inhibition than PLD activation and therefore it is possible that two G proteins and/or two divergent pathways are involved in neutrophil activation. A recent report suggests that neutrophil PLD is activated by a membrane associated small molecular weight G protein of the Rho family [92]. It is not known whether FPR (or any GPCR) interacts directly with any of the small G proteins of the ras super-family.

There is increasing evidence that the pathways involving pertussis toxin sensitive activation of phospholipase C (PLC-β isozyme) involves Gβγ subunits [67,93-95], whereas in pertussis toxin-insensitive activation of PLC, appears to be mediated by Gα subunits of Gq family [96]. In fact when FPR was transfected into COS-7 cells along with G14α or G16α (member of the Gq family) there was

an agonist dependent activation of the PLC-β2 isozyme (Melvin Simon, personal communication). Since neutrophil's Gq type G proteins constitute less than 1% of the total Gi2 amount (Simon, personal communication), the *in vivo* functional consequences of FPR-Gq interaction can only be speculated. Moreover, G16α expression is down regulated by 90% while Gi2α expression is increased by 160% when HL60 cells are induced to differentiate into neutrophil like cells with DMSO [97]. In this work authors concluded that "the pattern of G protein gene expression in this cell line may indicate that G16α is involved in regulating signal-transduction process that are not active in neutrophils but are manifested in other classes of leukocytes or in hematopoietic precursor cells." Clearly, many additional studies need to be carried out to delineate the role of various components involved in the generation of second messengers in neutrophils and the relative importance and roles of each of the second messenger pathways.

Desensitization

The term desensitization is used in a functional manner to describe a physiological end-point and does not imply in itself a specific mechanism. Desensitization may be defined as a general biological phenomenon characterized by waning of a physiological response, despite the presence of a stimulus. Two types of desensitization have been described. Homologous desensitization occurs when only the function of the occupied receptor is diminished (e.g. only beta

adrenergic receptors (βAR) are desensitized by the βAR-agonist isoprenaline). Heterologous desensitization occurs when occupancy of a distinct receptor results desensitization of another receptor pathway. Heterologous desensitization is thought to involve common second messengers for the two receptors (see [98] for a review).

Didsbury and collegues [99] have reported a novel type of desensitization for neutrophil chemoattractant receptors and termed such a process "receptor class desensitization." The authors' conclusions are based on the observations that C5a or fMLF ligands efficiently desensitized each other's receptors. Since C5a and fMLF do not bind to each other's receptors, this process differs from homologous desensitization which requires that only the agonist occupied receptors lose function. Moreover, at high doses of chemoattractants (> 100 nM fMLF or C5a) there was no heterologous desensitization effect on other calcium-elevating (a common second messenger) receptors (eg., α1-adrenergic receptors or P2-purinergic receptors on neutrophils.

Several distinct mechanisms have been described that mediate the phenomenon of desensitization. The **first** involves the uncoupling of receptors from G protein. Phosphorylation of serines and threonines near the COOH terminus and on the third cytoplasmic loop of some G protein coupled receptors results in the binding of a regulatory molecule arrestin (or its homologue) to the phosphorylated rhodopsin or βAR (reviewed in [100]). Phosphorylation and

arrestin homologue binding has been shown to cause functional uncoupling of rhodopsin and βAR from their signal transduction partners Gt and Gs respectively [98,101]. A **second** mechanism of desensitization is "sequestration" in which receptor's access to signal transducing machinery is blocked. Sequestration as proposed by Sibley and Lefkowitz [102] was relatively ambiguous. It mostly refers to internalization and separation from transduction apparatus. However, surface sequestration also occurs. Jesaitis et al [103], proposed that internalization was unnecessary for FPR desensitization as the receptors and G proteins are initially separated in the plane of the membrane, and removal to internal compartments comes later. The lateral segregation of proteins involved in signal transduction may or may not be unique to chemotactic receptors. In case of desensitization due to internalization, the receptors may be recycled back to the surface for subsequent activation. Laterally segregated receptors could potentially be released for participation in signal transduction events. The **third** mechanism of desensitization is down regulation in which internalized receptor is degraded following a trigger [49].

Biochemical Analysis of FPR-G Protein Complexes

G protein involvement in signal transduction generates a great deal of scientific interest because of many physiological and pharmacological events that are regulated by these pathways. Delineating the molecular basis of signal

transduction has been the primary focus for the past decade or so. In the last five years molecular biology aided the process by providing the chemical identities of the major players in G protein-mediated events. To avoid interference and cross talk from various cellular processes, in vitro experiments involving purified proteins are commonly used to study selected biochemical pathways in detail. Although reconstitution of purified transmembrane proteins into phospholipid vesicles have greatly aided the study of functional interaction of the components involved in G protein coupled pathways, reconstitution of such a system in detergents remains one of the ways the molecular complexes can be discerned [104].

Sedimentation Analysis of Proteins

Rate zonal ultracentrifugation, or velocity sedimentation, can be used to separate macromolecules according to their size and density. Particles can be subjected to very high angular acceleration In an ultracentrifuge for extended time periods. The increase in magnitude of angular acceleration as a function of radius (r^2) may be compensated by employing a gradient medium of increasing density and viscosity from top to bottom such as a sucrose or glycerol. The material most commonly used to form density gradients is sucrose because of its purity, low cost, and low interference with most chemical, enzymatic, and optical assays. The current standard of 5%-20% linear sucrose density gradient was empirically selected to impart an optimum balancing of the increasing centrifugal force at large

radius, thus resulting in uniform velocity for the migrating particles. Such a gradient is called an isokinetic gradient. A 5-20% sucrose gradient in a Beckman SW55 rotor appears to behave like an isokinetic gradient [106]. The density of the medium increases by 6% (from 1.015 mg/ml to 1.077 mg/ml) and viscosity increases by 65% (from 0.907 to 1.493) as the sucrose percentage (wt/wt) increases, in a linear sucrose density gradient, from 5% to 20%. The gradient also minimizes convection from density elevation due to sample zones and stabilizes against thermal mixing as well as minimizes mechanical disturbances, especially after a run. Velocity sedimentation in linear sucrose density gradients is a reliable method to characterize mixtures of macromolecules.

Theory of Sedimentation

A particle spinning in a centrifuge experiences a centrifugal force Fc. The magnitude of Fc depends on the mass of the particle, m, the distance from the center of rotation, r, and the angular velocity of the tube in which the particle is being spun.

$$Fc = m\omega^2 r$$

The particle displaces the medium and experiences a buoyant force, which results from the weight of the medium to displaced by the particle. This buoyant force reduces the net force on the macromolecule by $\omega^2 r$ times the mass of the displaced solution. The mass of the displaced solute is the volume of the particle multiplied

by the density, φ (g/cc) of the solvent. The particle volume is $m\bar{U}$, in which $\bar{U}$ (cc/g) is the partial specific volume of the particle, so that the buoyant force may be defined as is $\omega^2 r m \bar{U} \varphi$. As the particles move through the solvent, both the solvent and the particle experiences frictional force and the magnitude of this force is proportional to the difference between the velocities of the particle and of the solvent molecules as well as the shape of the particle. A filamentous particle presents more friction than a solid sphere of the same mass, and thus the shape of the particle in solution must be considered when interpreting its velocity of sedimentation. The hydrodynamic shape and the frictional drag is expressed as a frictional coefficient, f. The frictional force is expressed as $f\vartheta$, in which f is the frictional coefficient and ϑ is the net velocity of the particle relative to the centrifuge cell which holds the solvent. When the buoyant force and the frictional force are equal the the particle reaches terminal velocity and the particle sediments with uniform velocity and it is given by,

$$\vartheta = \omega^2 r m (1-\bar{U}\varphi) / f$$

The following corollaries can be drawn from the above equation:

1) a denser particle tends to move faster than a less dense particle

2) the denser the solution the more slowly the particle will move

3) the greater the frictional coefficient, the more slowly the particle will move.

These statements constitute the basic rules of velocity sedimentation and apply to all particles including roughly spherical compact particles like globular proteins.

Since the velocity of a molecule is proportional to the magnitude of the centrifugal field (i.e., $\omega^2 r$), it is a common practice to discuss sedimentation properties in terms of the velocity per unit field, or

$$s = \vartheta / \omega^2 r = m(1-\bar{U}\varphi) / f$$

where s is the sedimentation coefficient. The unit of s is seconds. Most s values are between 10^{-13} and 10^{-11} seconds. The value of s is usually reported in Svedberg units (S), where 1 Svedberg = 10^{-13} second. The S values of many proteins range from 1-10, while that of typical nucleic acids range from 10-100. A particle whose s value is 4 svedbergs is usually called a 4 S particle. Membrane proteins are often glycosylated and have significant amounts of detergent bound to them and behave anomalously. For these reasons the relationship between sedimentation coefficient and molecular mass of membrane proteins is neither linear nor simple.

Physicochemical properties of human neutrophil FPR have been characterized in digitonin and Triton X-100 [20,78]. FPR exhibits a high partial specific volume of 0.88 and 0.83 (cc/g) in Triton and digitonin respectively, suggesting extensive binding of detergent and/or endogenous lipid. Soluble proteins, free of detergent typically have a partial specific volume of 0.75 cc/g. In the studies mentioned above [20,78] guanine nucleotides were not employed suggesting that the species represented a receptor-G protein complex. Jesaitis et al [105], reported that photoaffinity labeled, octyl glucoside (1-octyl-β-D-

glucopyranoside or OG) solubilized FPR obtained from unstimulated cells exhibits two size forms with apparent sedimentation coefficients of approximately 4 and 7S. The faster sedimenting 7S form could be converted to a lighter 4S form by inclusion of GTPγS in the solubilization buffer, suggesting that the 7S form represented a complex between FPR and G protein. The interpretation of the S values in terms of molecular mass will be discussed in chapter 7.

Goals of This Dissertation

The primary goals of this dissertation are a) to demonstrate that the 7S species described above represented a physical complex between FPR and Gi2, b) to analyze the physical variables that dictate the formation of a stable and physiologically relevant 7S complex, and c) to determine the regions on FPR that are important for complex formation with G protein. The ability to resolve G protein free-FPR from FPR-Gi2 complex by velocity sedimentation formed the basis of all the experiments described in this dissertation. Such an assay also provided a unique opportunity to conduct synthetic receptor-mimetic peptide competition studies to study the interfacial contact sites between FPR and Gi2 protein.

Evidence is presented in chapters two and three to support the hypothesis that the 7S representes a physiologically relevant signal transducing entity containing FPR and Gi2 protein. Chapters four and five describe approaches taken

to address questions about the integrity and interacting regions responsible for formation of FPR-Gi2 complex. The findings using FPR peptides, reported in this dissertation, highlight the differences between direct analysis of receptor-G protein binding versus G protein activation (using functional assays). Experiments involving functional assays contribute information towards understanding receptor-G protein coupling. Results presented in this dissertation on the molecular association between FPR and G protein may set a new criterion to permit additional conclusions to be drawn when coupled with molecular genetic and functional assays.