



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

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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 Gi α .

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.

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Rajani Kanth Bommakanti

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

of

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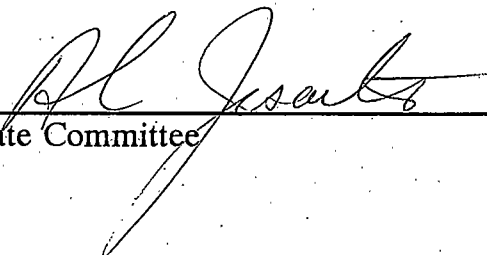
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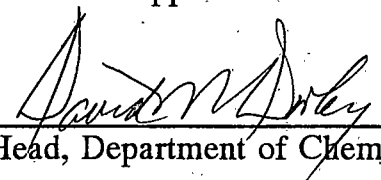


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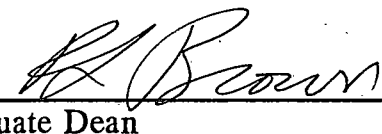


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SignatureRajani BonmakantiDateApril 22, 1994

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.

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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 Gi α .

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 transducin (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-N ϵ -(2-(p-azido[¹²⁵I]salicyl-amido)ethyl-1,3'-dithiopropionyl). This specific FPR labeling reagent is abbreviated fMLFK-[¹²⁵I]ASD, and was also formerly referred to as FMLPL-[¹²⁵I]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 polypeptide 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 transfection 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 β 2-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 helices. 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 identity (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)

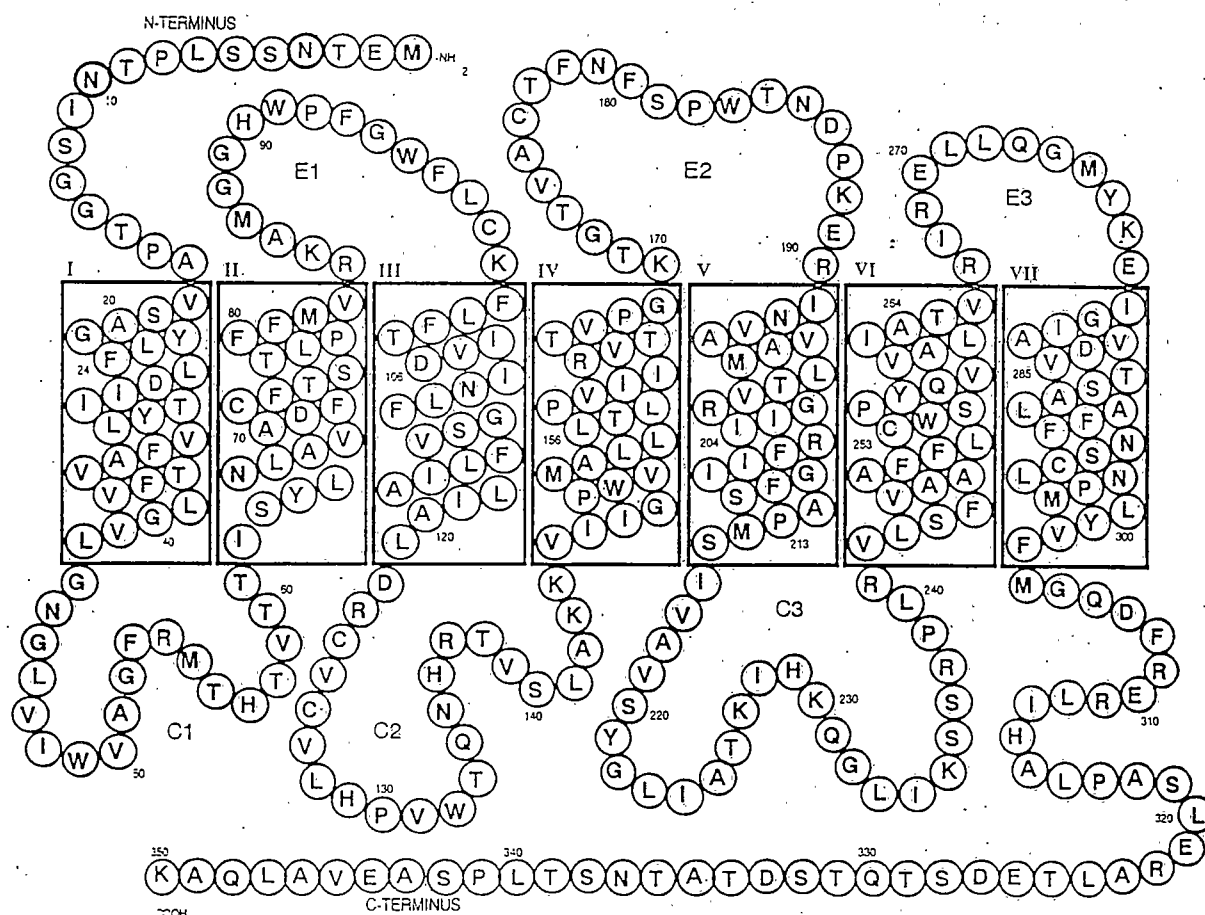


Figure 1: Schematic model of the FPR polypeptide chain in the neutrophil plasma membrane. I through VII represent the putative lipid bilayer spanning domains, which are connected by loops E1, E2, and E3 on the extracellular side and loops C1, C2, and C3 on the cytoplasmic side of the membrane. The asparagine 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 A₂ 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 paradigm 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 extracellular 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 binding 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 K_d 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]. $G\alpha$ binds guanine nucleotides and has intrinsic GTPase activity where the terminal phosphate on bound GTP is cleaved to form GDP. In its basal state $G\alpha$ contains bound GDP and is tightly associated with the $\beta\gamma$ 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 $\beta\gamma$ subunit complex greatly stimulates interaction between $G\alpha$ and the receptor, and the nucleotide exchange (reviewed in [56]). Moreover, there is now evidence that the $\beta\gamma$ subunits also contribute to the specificity of receptor-G protein coupling [57]. Upon activation, *in vitro*, $G\alpha$ -GTP dissociates from the $\beta\gamma$ subunits (see Figure 2). Both species that form after receptor stimulation ($G\alpha$ -GTP and $\beta\gamma$) 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 $G\alpha$ subunits have been cloned and sequenced. The $G\alpha$'s are divided into four subfamilies G_s , G_i , G_q and G_{12} based on sequence homology. Molecular weights of the $G\alpha$ 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 $\beta\gamma$ subunits are present [60]. Several of the $G\alpha$ 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 $G\alpha$ are myristoylated) may be important in the membrane attachment of $G\alpha$. 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 $\beta\gamma$ subunits are much more hydrophobic than the α subunit and have a strong tendency to attach to phospholipid bilayers, and with each other. The $\beta\gamma$ 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 $\beta\gamma$ subunits in signal transduction have been recently reviewed in [66-68].

Currently, seven different $G\gamma$ 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 $G\gamma$ subunits also differ in their modification by prenyl groups: $G\gamma 1$ is farnesylated; whereas $G\gamma 2$ 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.

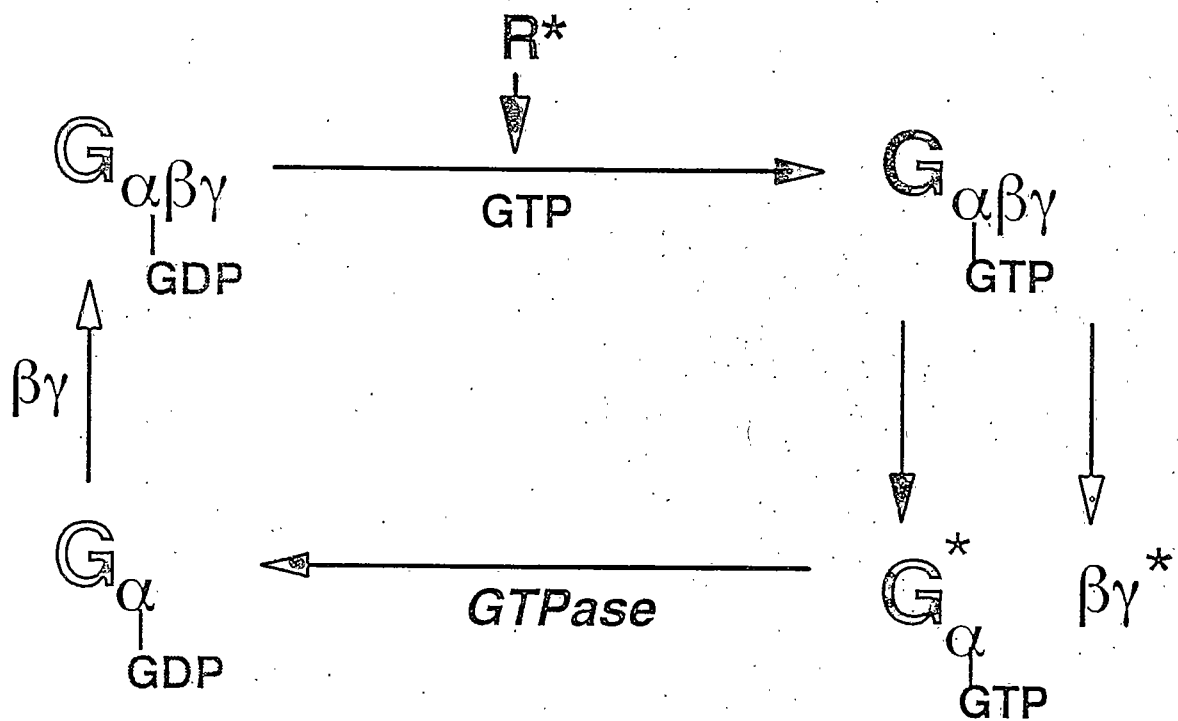


Figure 2: Diagram depicting the activation and deactivation cycle of G proteins. $G\alpha\beta\gamma$, R^* represents the heterotrimeric G protein, and agonist occupied G protein-coupled receptor.

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 GTP γ S) inhibits high affinity binding of fMLF to FPR with an IC₅₀ 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 GTP γ S with an IC₅₀ 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 G α 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 GTP γ S binding to G α) [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 $G\alpha$ or $\beta\gamma$ 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 superfamily.

There is increasing evidence that the pathways involving pertussis toxin sensitive activation of phospholipase C (PLC- β isozyme) involves G $\beta\gamma$ subunits [67,93-95], whereas in pertussis toxin-insensitive activation of PLC, appears to be mediated by G α subunits of Gq family [96]. In fact when FPR was transfected into COS-7 cells along with G14 α or G16 α (member of the Gq family) there was

an agonist dependent activation of the PLC- β 2 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, G16 α expression is down regulated by 90% while Gi2 α 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 G16 α 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 (β AR) are desensitized by the β AR-agonist isoprenaline). Heterologous desensitization occurs when occupancy of a distinct receptor results in 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 colleagues [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., α 1-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 β AR (reviewed in [100]). Phosphorylation and

arrestin homologue binding has been shown to cause functional uncoupling of rhodopsin and β AR 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 F_c . The magnitude of F_c 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.

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

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

$$s = v / \omega^2 r = m (1 - \bar{v} \rho) / f$$

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 GTP γ S 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 represents 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.

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CHAPTER 2

RECONSTITUTION OF A PHYSICAL COMPLEX BETWEEN THE N-FORMYL CHEMOTACTIC PEPTIDE RECEPTOR AND G PROTEIN: INHIBITION BY PERTUSSIS TOXIN-CATALYZED ADP RIBOSYLATION

Introduction

The binding of N-formyl peptides to specific cell surface receptors [1-3] on human leukocytes results in the activation of a variety of cell functions, including chemotaxis, lysosomal enzyme secretion, and superoxide production [4]. There is a substantial body of evidence suggesting that the receptor for these peptides mediates transduction through interaction with a guanyl nucleotide binding-protein or G protein [5-7]. The N-formyl chemotactic peptide receptors in membranes [8] or permeabilized neutrophils [9] exist in a high affinity state for agonist which can be specifically converted to a low affinity state by guanyl nucleotides. It has also been shown that GTP γ S inhibits high-affinity binding of fMet-Leu-[³H]Phe to fMLF receptors with an EC₅₀ value of about 20 nM [10].

We have recently [11] presented data which indicate that the octyl glucoside-solubilized N-formyl chemotactic peptide receptor from unstimulated neutrophils exhibits two forms with sedimentation coefficients of approximately 4S and 7S. The 7S form could be converted to the 4S form by GTP γ S with an EC₅₀ value of about 20 nM, suggesting that the 7S form represented a receptor-G protein complex. The inhibition of high-affinity agonist binding to G protein coupled-receptors by guanyl nucleotides is generally accepted to be indicative of uncoupling of receptor and G protein [5]. The similar GTP γ S concentration dependence of the 7S to 4S conversion [11] and the inhibition of high-affinity agonist binding by GTP γ S [10] further substantiates that the 7S form of the fMLP-receptor represented the G protein-coupled form.

Pertussis toxin-catalyzed ADP-ribosylation of a 40 kDa membrane substrate in neutrophils also disrupts the functional coupling of the N-formyl chemotactic peptide receptor to neutrophil activation [12]. The cholera toxin induced ADP-ribosylation of this substrate in membranes can be modulated by N-formyl peptide binding [13,14], suggesting an interaction with the ligand occupied receptor. Finally, chemotactic N-formyl peptides stimulate guanyl nucleotide binding and pertussis toxin-sensitive GTPase activity in the neutrophil membranes [15,16]. The molecular mechanisms of the uncoupling however, remains unclear.

Pertussis toxin substrates of 40 kD and 41 kD in neutrophils and HL60 cells have been purified and appear to be identical to Gi2 and Gi3 [17-19]. Gi2

(also termed G_n) has been shown to be the major substrate in mature human neutrophils [20] and HL60 cells [18,21]. Both G_{i2} and G_{i3} have been demonstrated to be functionally coupled to N-formyl chemotactic peptide receptors in HL60 cells [21]. Several studies have demonstrated that high affinity formyl peptide binding, formyl peptide-stimulated GTPase activity, and formyl peptide-mediated activation of phospholipase C [22,23] can be restored by rat or brain G_i / G_o proteins. These various criteria clearly indicated that restoration of function of receptor-G protein coupling can be demonstrated. To date, however, there has been no demonstration of the formation of a physical complex of the N-formyl chemotactic peptide receptor with a GTP binding protein(s).

Active functional receptor G protein complexes have clearly been demonstrated in rhodopsin, β -adrenergic, muscarinic, dopamine, and adenosine receptor systems among the many now investigated [24]. In nearly all cases, evidence for such association has been the GTP sensitivity of agonist binding (or phototransformation in the case of rhodopsin) or agonist/light-induced stimulation of the GTPase activity. In a number of detergent-based systems [25-28] used for the study of such interactions, including the formyl peptide receptor system [11,29], receptors or G proteins have been shown to be released from apparent complexation upon exposure to GTP or its nonhydrolyzable derivatives. Reconstitution of the functional activities described above have also been demonstrated in both membrane and detergent based systems. To our knowledge

no studies have reported direct measurement of such complexes by hydrodynamic analysis of solubilized radiolabeled receptors.

In this report, we provide evidence for the direct formation of a 7S complex between 4S receptors and either pure bovine brain Gi or purified endogenous neutrophil Gn. We additionally show that stoichiometric ADP-ribosylation of Gi by pertussis toxin prevents the reconstitution of 7S complexes with G protein. Moreover, the structurally related G protein of the visual system, Gt, doesnot reconstitute a 7S complex, indicating specificity of the receptor for Gn and Gi. These observations suggest that the 7S complexes are the result of physical association of receptor and G protein while the 4S form is uncoupled from G proteins.

Materials and Methods

Buffers, chemicals, methods of cell preparation were as previously described [11,30]. Chemicals used for G protein isolation were as described by Bokoch et al [20,31]. Anti G protein antisera were prepared as described in [20].

Identification of 4S or 7S Forms of N-formyl Chemotactic Peptide Receptor:

Plasma membrane fractions were prepared from degranulated neutrophils as described previously by Parkos et al. [30]. The N-formyl chemotactic peptide receptors were specifically labeled with FMLPL-SASD [¹²⁵I] by the procedures

of Allen et al. [32,33]. In order to prevent isolation of endogenous 7S receptor forms during the membrane solubilization and reconstitution step, the labeled membranes were treated with 10 μ M GTP γ S and were subsequently washed with "Relax buffer," (0.5-1 mg/ml) in 10 mM Hepes pH 7.4, 100 mM KCl, 10 mM NaCl, 1 mM EDTA, 1 mM PMSF, 10 μ g/ml chymostatin, containing 1.0 M NaCl. Labeled membranes were pelleted and resuspended at 0.5-0.8 mg protein/ml in the Relax buffer containing 1.0% 1-octyl- β -D-glucopyranoside (octyl glucoside), then incubated on ice for 2 hours. Insoluble material was removed by sedimentation at 45,000 rpm in a Beckman 60Ti rotor for 30 min at 4°C. Supernatant extracts (100 μ l) were layered onto 5 ml, 5-20% linear sucrose density gradients prepared in the extraction buffer. Gradients were centrifuged in an SW55 Beckman swinging bucket rotor for 16 hours at 45,000 rpm and fractionated into 13 x 400 μ l fractions. Alternatively, to conserve material and reduce sedimentation time, experiments were also carried out using 25 μ l samples on 0.7 ml gradients sedimenting for 8 hours in the same rotors. In some cases fractions were diluted 1:10 in extraction buffer, concentrated to the original volume, and resedimented by the first procedure. Sedimentation was calibrated with known protein standards by centrifuging a mixture containing 25 μ g each of cytochrome C (2.1S), BSA (4.4S), porcine immunoglobulin (7.7S), and bovine catalase (11.2S) in parallel with the experimental gradients. Photoaffinity labeled receptor content was measured by autoradiography of receptors separated on SDS-

PAGE or by ^{125}I content of fractions as described previously [11].

Reconstitution of Receptor with G Protein and Analysis:

Octyl glucoside extracts were divided into individual incubation mixtures with the indicated levels of G protein under the conditions described in the text. Incubations were carried out for 2 hours on ice. Samples were then layered onto 5 ml 5-20% linear sucrose gradients and receptor sedimentation analyzed as described above. The G protein concentration dependence of the reconstitution of the 7S form of the receptor was fitted with the Hill equation using a nonlinear non-weighted curve-fitting computer program [34]. Endogenous G protein did not couple to receptor as evidenced by the identical sedimentation pattern at 0 G protein in the presence of $10\mu\text{M}$ $\text{GTP}\gamma\text{S}$.

Preparation of G Proteins:

G_i was purified from bovine brain as described in [35] and resolved from G_o by chromatography on a 20 ml DEAE Sephacel column equilibrated with 25 mM Tris-HCl pH 8, 1 mM EDTA, 1 mM NaCl, 0.6% lubrol, and eluted with a linear gradient (200 ml) of 0-250 mM NaCl in the same buffer. Purity was judged to be 95% or higher as assessed by SDS-PAGE and silver staining. Concentrations given for the G proteins are based on the protein concentration determined after the final elution step. The G proteins used were functionally

intact as determined by GTP γ [^{32} S] binding [20]. Neutrophil Gn (or Gi2) was isolated as described by Bokoch, et al. [20,31] up until the heptylamine sepharose chromatography, with the exception that purifications were performed in the absence of AlCl₃, MgCl₂, and NaF. The Gn was further purified to apparent homogeneity by DEAE-Sephacel chromatography as described above, concentrated by hydroxyapatite chromatography and switched into Lubrol-containing buffers by G25 gel filtration, as described in [36]. Functional bovine transducin able to stabilize the M-II form of rhodopsin, was prepared by the method of Stryer et al. [37] and was the kind gift of Dr. Heidi Hamm.

ADP-ribosylated Gi was prepared by incubation of 350 μ g Gi with 20 μ g/ml pertussis toxin in 100 mM Tris HCl pH 8, 1 mM EDTA, 2.5 mM MgCl₂, 10 mM thymidine, 1 mM ATP, 0.1 mM GTP, 0.5 mM dimyristoyl phosphatidylcholine, 10 mM dithiothreitol, and 2 mM NAD for 30 minutes at 30°C. The level of ADP-ribosylation of the Gi was assessed with a parallel incubation in the presence of [^{32} P]NAD (specific activity = 300 cpm/pmole) that was analyzed by TCA precipitation/filtration, as in [36], as well as by subsequent [^{32} P]NAD labeling of the Gi pre-ribosylated with unlabeled NAD (see Fig. 4 inset). Determination of [^{35} S]GTP γ S binding to Gi and amido black protein analyses were as described in Bokoch et al [36].

Results

In detergent (Triton X-100 and digitonin), the covalently liganded N-formyl chemotactic peptide receptor from human neutrophils behaves as a monodisperse species of ~63 kDa upon sedimentation equilibrium analysis [38]. The receptor from differentiated HL60 cells elutes in deoxycholate as a ~66 kDa species upon gel filtration [29]. These estimates are consistent with the apparent size of the affinity labeled receptor upon reduced SDS-PAGE, where the liganded receptor migrates as a broad band of 50-70 kDa [38-40]. It has been demonstrated that the octyl glucoside-solubilized N-formyl chemotactic peptide receptor retains the capability for interaction with endogenous neutrophil G protein(s) upon reconstitution into phospholipid vesicles [41].

We have previously shown that the photoaffinity labeled, octyl glucoside-solubilized N-formyl peptide receptor from human neutrophils exhibits two forms upon sucrose density gradient sedimentation, with apparent sedimentation coefficients of approximately 4S and 7S [11]. The 7S form could be converted to the 4S form by GTP γ S with an EC₅₀ of ~20 nM, and this size change in the receptor appeared to correlate with a reduction in sedimentation rate of Gn alpha and beta subunits. These data suggested that the 7S form of the N-formyl chemotactic peptide receptor might represent a physical complex with endogenous Gn protein [11].

To show that the 7S form is indeed a complex of 4S receptor and Gn, we

attempted its reconstitution with purified Gn [20,31]. The 4S form of the photoaffinity-labeled receptor isolated from GTP γ S-treated neutrophil membranes by octyl glucoside extraction and was incubated with different concentrations (10-600 nM) of purified Gn. Each incubation was subsequently analyzed on a velocity sucrose density gradient, and a representative experiment is shown in Figure 3. It can be clearly seen that there is a major shift of receptors (identified autoradiographically [11] and quantitated densitometrically) from the 4S form (peak fraction 4) to the 7S form (peak fraction 7). A small percentage of receptor (7-8%) did not undergo the shift. The reconstituted 7S receptor form was fully sensitive to guanyl nucleotides, as no such shift was detectable in the presence of GTP γ S (see Figure 4). In addition the reconstituted 7S form could be reversed to the 4S form when incubated with GTP γ S. Resedimentation of the peak or flanking fractions from the zero G protein condition resulted in distributions virtually indistinguishable (not shown) from the original profile, suggesting that the distributions was representative of unassociated receptor.

SDS-PAGE analysis of these gradients confirmed the presence of Gn in these 7S fractions (not shown) as had been previously shown by western blot analysis of the endogenous 7S complexes [11]. Moreover, when 1 part in 8 anti-G protein antisera [11,12] was included in similar gradients and a reconstitution mixture containing 300 nM Gi, about 30% of the receptor in the 7S fraction was shifted into fractions 9S and higher, while less than 1% was shifted when equal

amounts of prebleed control serum were added. Together these results suggest that the 7S form of the N-formyl chemotactic peptide receptor may be a physical complex of receptor and G protein.

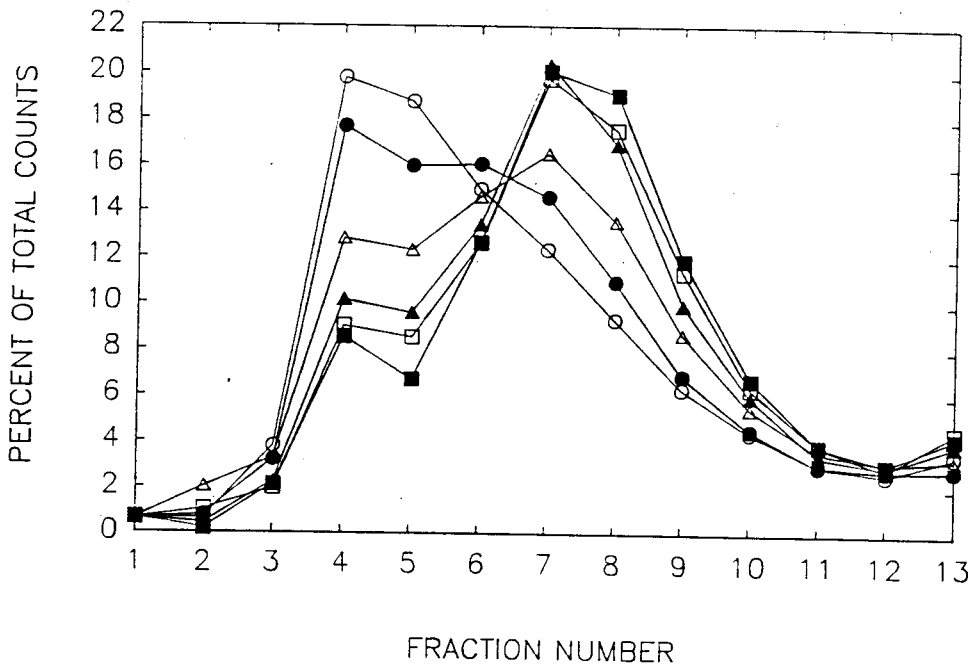


Figure 3. Reconstitution of 7S N-formyl chemotactic peptide receptor complexes with purified human neutrophil Gn. Octyl glucoside-extracted, photoaffinity-labeled N-formyl chemotactic peptide receptor was incubated with different concentrations of Gn as described in Materials and Methods. The mixtures were sedimented in an ultracentrifuge for 16 h at 135,000 $\times$ g and fractionated into 13 equal fractions. The receptor content of each fraction evaluated densitometrically, is plotted as a function of fraction number. The 4S and 7S migration distances correspond to fractions 5 and 7, respectively. The concentrations of Gn were 0 (○), 10 (●), 100 (△), 200 (▲), 400 (□), and 600 nM (■).

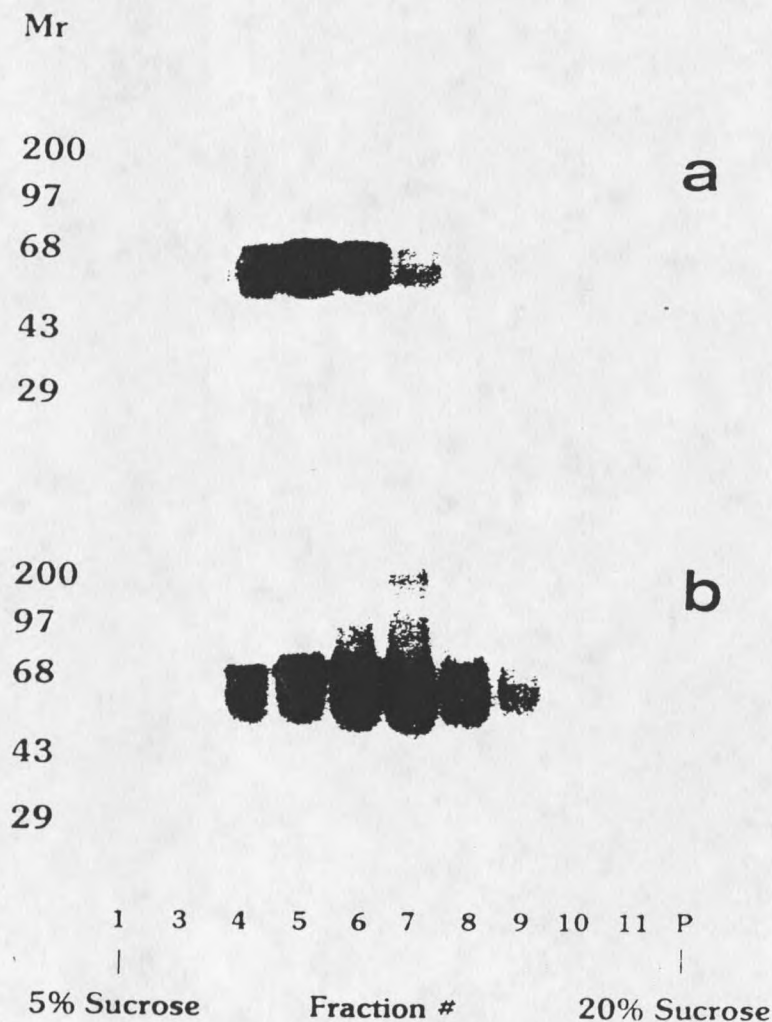


Figure 4. Autoradiograms of ^{125}I affinity labeled and reconstituted formyl chemotactic peptide receptor separated on sucrose density gradients. (A) Fractions from the density gradients containing the reconstitutions with 0 nM (a) and 200 nM Gn (b) shown in Figure 1 were solubilized in SDS and run on 9% SDS polyacrylamide gels. The gels were dried and developed for autoradiography after seven days of exposure with Du Pont Cronex Quanta III intensifying screens as described originally by Allen et al. [32]. The formyl peptide receptor is observed as a broad species between the 68 and 43 kDa Mr markers in both 2a and 2b. Also detectable is the presence of a nonspecifically labeled SASD-binding protein of Mr = 68 kDa, which serves as an internal sedimentation standard (3.5S) and is variably present in membrane batch and ligand age.

Autoradiographic analysis of SDS-PAGE gels of each fraction of these runs confirmed that a major portion of the radioactivity measured was derived from receptor. Figure 4 shows the shift in sedimentation position of the 4S receptor to the 7S reconstituted receptor-G protein complex at a Gn concentration of 200 nM. The two autoradiograms indicated that the receptor was shifted in position (not Mr) in the sedimentation profile and relative to the "internal standard" of nonspecifically labeled 68 kDa SASD-binding protein [32,33], the amount of which varied in the preparation depending on the age of the ligand and membrane batch used.

To evaluate the selectivity of the receptor for its endogenous G-protein transduction partner, Gn vs. Gi proteins from other sources, a similar analysis was performed using bovine G proteins, Gi purified from brain (consisting of Gi1 and Gi2 [42], and transducin, Gt from retina. Averaging of the results from multiple sedimentation runs permitted us to estimate the amount of brain Gi or neutrophil Gn necessary to convert fifty percent of the 4S receptor form to the 7S form (Figure 5).

The EC_{50} for reconstitution of the 7S form of the receptor was 170 ± 40 nM for Gi and 70 ± 25 nM for Gn based on a computer calculated fit of the data to the Hill equation [34]. Functionally active bovine transducin (Gt) was incapable of complexing with the formyl peptide chemotactic receptor in this system, as it showed no shift in receptor sedimentation rate even at 5 μ M added transducin.

It is noteworthy that the EC_{50} for the reconstitution of the 7S form of the receptor-Gi protein complex was the same when using receptors obtained from membranes of unstimulated neutrophils and were compared to receptors obtained from agonist stimulated cells.

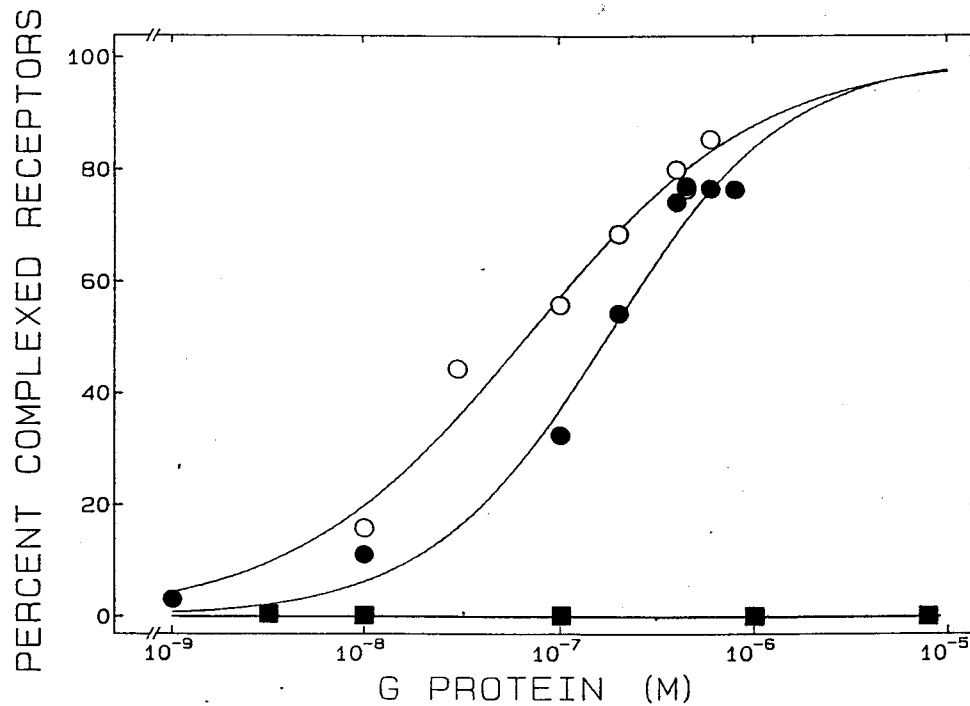


Figure 5. Quantitative comparison of the ability of Gn, Gi, or Gt to reconstitute a N-formyl peptide receptor G protein complex. Reconstitution analyses as described in Figure 1 were used to determine the avidity of N-formyl peptide receptor for G-protein by measuring the increase in 7S form as a percentage of the saturation value observed with excess G-protein. O, Gn (n=3); ●, Gi (n=6); ■, Gt (n=2).

Evidence for the functional significance of the reconstitution of the physical interaction of receptors and G proteins is obtained by determining the ability of the receptor to reconstitute with ADP-ribosylated G protein. Pertussis toxin-catalyzed ADP-ribosylation of G_i proteins is known to uncouple the G_i protein from their receptor transduction partners [5]. This uncoupling has been demonstrated by examining receptor-stimulated functions in intact cells, membranes or reconstituted systems, including phospholipase C activation [23], adenylate cyclase inhibition [43], receptor-stimulated GTPase activity [16,44], high affinity agonist binding to the N-formyl chemotactic peptide receptor [16], and by other means [44,45]. However, physical uncoupling of receptor and G protein by ADP-riboyslation has not, to our knowledge, been demonstrated. Indeed, one can postulate that functional uncoupling could take place without physical disruption of the R-G complex. We therefore examined the ability of ADP-ribosylated G_i to reconstitute the N-formyl chemotactic peptide receptor-G protein (7S) complex.

Table 1 documents the preparation of ADP-ribosylated G_i . The protein was ADP-ribosylated to an extent of 0.96 pmoles of ADP-ribose/pmole of GTP γ S binding, as determined by direct analysis of [^{32} P]NAD incorporation. That the protein was totally ADP-ribosylated is also shown by the inability to significantly label with [^{32} P]NAD subsequent to initial ADP-ribosylation with unlabeled NAD (Inset, Figure 6). In order to confirm that the ADP-ribosylated G_i was still

functional after the ADP-ribosylation reaction, we determined (see Table 1) that GTP γ S binding to the ribosylated Gi reached a level of 0.9 pmol GTP γ S/pmol protein; the same value as was obtained with the non-ribosylated (control) protein.

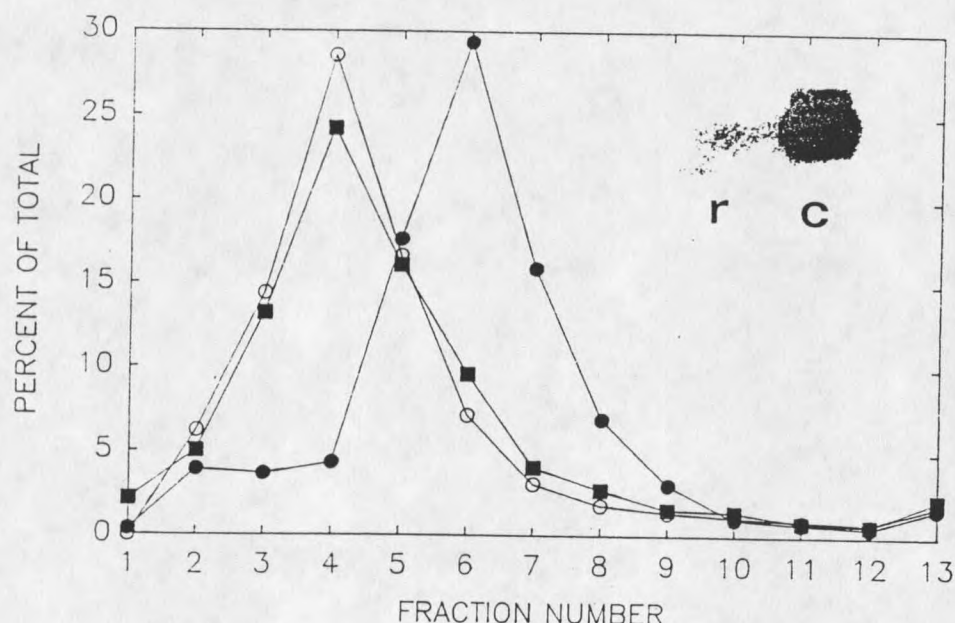


Figure 6. ADP-ribosylated Gi is incapable of forming physical complexes with N-formyl peptide receptor. Reconstitution of receptor was attempted with 400 nM Gi + 10 μ M GTP γ S (O), 400 nM Gi (●), and 3000 nM ADP-ribosylated Gi (■) as performed in Figure 1, except that this sedimentation was performed in smaller tubes for shorter times (8 hours). Percent of the total sedimentable radioactivity in the gradient is plotted as a function of fraction number of the octyl glucoside-containing velocity sucrose gradient. Inset shows the labeling of either control (C) or pre-ADP-ribosylated Gi (R) (as described in Table 1) with [^{32} P]NAD and pertussis toxin. The autoradiogram was overexposed to demonstrate that all of the Gi protein was stoichiometrically ADP-ribosylated by the initial (cold) ADP-ribosylation reaction (See Materials and Methods).

Table I: Characterization of G_i ADP-ribosylation by Pertussis Toxin

Sample	Protein (pmol/ml)	GTP γ S Bound (pmol/ml)	$\frac{\text{pmol GTP}\gamma\text{S}}{\text{pmol protein}}$	ADP-ribose Incorporated (pmol/ml)	$\frac{\text{pmol ADPr}}{\text{pmol protein}}$	$\frac{\text{pmol ADPr}}{\text{pmol GTP}\gamma\text{S}}$
Control G _i	0.495	0.470	0.95	---	---	---
ADP-r G _i	2.66	2.34	0.88	2.24	0.84	0.96

As shown in Figure 6, when the stoichiometrically ADP-ribosylated Gi protein was used to attempt reconstitution of the N-formyl chemotactic peptide receptor-G protein (7S) complex, we were not able to shift the 4S receptor to the faster sedimenting form. Even though we raised the concentration of ADP-ribosylated Gi to 20 times the concentration that resulted in 80% complexation of receptor by control Gi, receptor sedimentation profiles showed no sensitivity to GTP γ S, indicating no conversion receptor to the 7S form. These data suggest that the pertussis toxin-catalyzed ADP-ribosylation of G proteins prevents their physical complexation with receptors. This result also suggests that previous studies showing only partial uncoupling, by the 14 amino acid peptide mastoparan as a receptor analog [40] is not fully representative of complete receptor-G protein interaction.

Discussion

In this study, we demonstrated that the sedimentation rate of octyl glucoside-solubilized N-formyl chemotactic peptide receptor was increased when incubated with either the endogenous neutrophil pertussis toxin substrate, Gn, or purified Gi protein(s) from bovine brain but was unchanged when incubated with bovine transducin. These changes were prevented by guanine nucleotides and, in the case of bovine Gi, by pertussis toxin-catalyzed ADP-ribosylation. The most likely explanation for these observations is that the 7S form of the receptor is a

physical complex containing the heterotrimeric G protein and receptor. Although it is conceivable that such a change could result from a G protein-dependent oligomerization of receptor with itself or other proteins, it is highly unlikely for several reasons. First, our results are consistent with the large body of biochemical evidence measuring functional parameters such as agonist binding, GTPase activity, and phototransformation observed in many receptor systems and interpreted as evidence for complexation of receptors and G-proteins [24]. Second, a G protein dependent oligomerization of receptors with other proteins would necessarily imply that the newly formed complex would have to interact with G-proteins again in the GTP-bound form in order to explain the sensitivity of the 7S form to guanyl nucleotides. Evidence for a second collisional interaction of receptor and G-protein does not exist. Finally, our data indicating specific immunosedimentation of receptor by anti-G protein antisera, suggests that stable receptor G-protein complexes have been formed.

The 4S and 7S forms of the N-formyl chemotactic peptide receptor appears relatively broad in Figures 2.1 and 2.2 (half band width approx. 35% of gradient) in contrast to other membrane proteins (half band width of 15-20%) [46,47]. Such broadening could arise from a number of sources including heterogeneous glycosylation of receptor [48], variable amounts of bound lipid and/or detergent on this very hydrophobic protein [38], or even the presence of oligomeric forms of the receptor [49], or other proteins [50]. Resedimentation of receptors in the

peak and flanking fractions of the 4S (zero G protein runs) indicate that the distributions do not narrow or shift in position, suggesting that these latter two possibilities are unlikely. Technical considerations such as anomalous zone broadening, wall effects, and diffusion [51] are sufficient to explain, since narrower distributions are observed (HBW = 12% of gradient) when shorter gradients in narrower tubes were used to shorten sedimentation time and reduce wall effects (Figure 6).

Our reconstitution evidence is also consistent with quantitative estimates of requirement for functional interactions within the cell. The N-formyl chemotactic peptide receptor was not purified in this work and we estimate that it was present in the starting membranes at a level of 5-10 pmol/mg membrane protein. Endogenous Gn protein was present at a level of ~1 nmol Gn α subunit/mg membrane protein [20,52]. Therefore, the amount of total Gn α subunit relative to receptor was approximately 100 to 1 in the native membrane. If one makes the assumption that only the $\alpha\beta\gamma$ complex of Gn is able to effectively interact with receptor, this ratio is reduced to 25 to 1, since $\beta\gamma$ subunits appear to be present at levels approximately one fourth of α subunits [20]. We induced approximately 80% of the maximum conversion of the labeled receptor from the 4S to 7S form (Figure 5) at 300 pmol/ml Gn. This level is similar to the level of the oligomeric Gn that is present in the native membrane. We are, not using a huge excess of G protein relative to what exists in the cell

to achieve the reconstitution of the R-G complex *in vitro*. In both *in vitro* and *in vitro* the G protein levels are considerably in excess (>100 fold) of the N-formyl peptide receptor itself.

The similar values for the EC_{50} for complex formation seen with the brain and neutrophil G proteins may reflect identical efficiency in coupling of Gn and the Gi2 portion present in the mixed brain Gi preparation. Our experiments, however, are not able to determine if receptor interacts with both Gi1 and Gi2 in the brain preparation. At least three polypeptide stretches of the alpha subunit of Gi2 and Gn (corresponding to residues 8-23, 311-328, and 340-350 of transducin) maybe be interacting with receptors based on analogy from peptide competition studies of the interaction of rhodopsin and transducin [53,54]. The alpha subunits of bovine Gi2 and human Gn are 100% identical in these stretches. The identity between the alpha subunits of Gn and Gi1 in these regions is 87, 100, and 100%, respectively. The overall identity of Gi1 and Gi2 alpha subunits, moreover is 90% suggesting the differences are rather small but significant. Transducin, which does not interact with the formyl peptide receptor, has a sequence that is only 44, 78, and 91% identical to Gn in the above polypeptide regions. Although these regions are 88, 83, 100% homologous, if conservative substitutions are considered, there appears to be insufficient similarity to allow transducin to interact with the human chemotactic receptor. These results therefore suggest that the 7S complex formation is selective and can recognize structural differences in

G proteins. Thus, the methods described in this report should be suitable for detailed studies of the specificity of interaction of the N-formyl peptide receptor with various other forms of G proteins.

The technique might also be applicable to investigations of functional modification of receptor. Results from our laboratory using labeled N-formyl peptide receptor prepared from unstimulated cells indicate that its interaction with G-protein is as avid as the receptor prepared from stimulated cells described in this study (data not shown). If it can be shown that the receptor from desensitized cells and unstimulated or stimulated cells are basically equivalent in their affinity for G-protein, then it would support the hypothesis that the lateral segregation of receptor from its transduction partner in the plane of the plasma membrane [11,55] may be sufficient to explain receptor specific desensitization in neutrophils.

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CHAPTER 3

CHARACTERIZATION OF COMPLEX FORMATION BETWEEN Gi2 AND OCTYL GLUCOSIDE SOLUBILIZED NEUTROPHIL N-FORMYL PEPTIDE CHEMOATTRACTANT RECEPTOR BY VELOCITY SEDIMENTATION

Introduction

The N-formyl peptide chemoattractant receptor (FPR) mediates the activation of neutrophils by interacting with a guanine nucleotide binding protein (G protein) [1-4]. Such activation ultimately leads neutrophils to generate free radicals which are implicated in microbicidal activity [5]. Hydropathy analysis of the primary sequence of FPR indicates that it has seven predominantly hydrophobic stretches which are characteristic of G protein-coupled receptors (GPCR) [6]. This analysis suggests that the polypeptide could wind in and out of the plasma membrane seven times with hydrophilic connecting loops on both the extracellular and the cytoplasmic sides of the membrane. Electron diffraction studies indicate that bacteriorhodopsin, a light-driven, non G protein-linked, proton pump contains a seven transmembrane helix motif [7,8] connected by relatively hydrophilic loops.

GPCR are thought to share structural similarities with bacteriorhodopsin and analogous topographic models have been proposed and have been partially validated for rhodopsin [9-11], and β 2-adrenergic receptor [12]. Based on sequence similarities between FPR and other GPCR, working models of the transmembrane organization of FPR have been proposed [6,13,14].

Physicochemical properties of human neutrophil FPR have been characterized in Digitonin and Triton X-100 detergent extracts [15,16]. FPR exhibits a high partial specific volume of 0.88 and 0.83 in Triton and Digitonin respectively, suggesting extensive binding of detergent and/or endogenous lipid. Jesaitis and coworkers reported that photoaffinity agonist-labeled, octyl glucoside (1-octyl- β -D-glucopyranoside) -solubilized FPR, obtained from unstimulated cells, exhibits two interconvertible size forms with apparent sedimentation coefficients of approximately 4S and 7S [17]. Rate zonal or velocity sedimentation, separates macromolecules according to their size and density. The faster sedimenting 7S form could be converted to a lighter 4S form by inclusion of GTP γ S in the solubilization buffer, suggesting that the 7S form represented a complex between FPR and G protein. We have recently shown that 4S FPR directly interacts with bovine brain Gi2 or endogenous neutrophil Gn [18] which is 95% identical to Gi2 [3]. FPR does not form a complex with Gt [18] or Gt/Go mixtures (unpublished observation). Silver staining, radioautography and immunoblotting analysis of sucrose gradient fractions containing the FPR-Gi complexes indicate that the G

protein subunits cosediment with the 7S FPR complex [17].

Further characterization of FPR-Gi2 complex formation is useful, since physical reconstitution can be used to study the structural determinants involved in the binding of FPR to G protein [6,19]. In this report, we investigated the biochemical variables that control the formation of the 7S FPR-Gi2 complex. Our results show that the FPR-Gi2 complex is disrupted by GDP β S which indicates that the guanine nucleotide site in the complex is empty. The empty nucleotide is a hallmark of a functional guanine nucleotide exchange intermediate. The reconstituted 7S species was stable under a variety of conditions including a pH 9-7, detergent environments below and above the critical micelle concentration (CMC), and salt concentrations from 0 to 200 mM. Formation of the 7S FPR-Gi2 protein complex did not require the presence of exogenous cofactors from neutrophil cytosol or Mg²⁺ ions. These results, along with our previous findings, indicate that sedimentation analysis of FPR-Gi2 complexes can be used to probe the molecular interactions of agonist-occupied chemotactic receptor with its signal transduction partner the heterotrimeric G protein Gi2.

Materials and Methods

Buffers, chemicals, methods of cell preparation were as previously described [17,20]. Chemicals used for G protein isolation were as described by Bokoch et al, [21].

Preparation of G Protein:

Gi was purified from bovine brain as previously described [22] with the following modifications. For the first two chromatographic steps DEAE-Sephacel was replaced by Q-Sepharose Fast Flow ion exchange media (Pharmacia) and Ultrogel AcA 34 was replaced by Sephacryl 200-HR gel filtration media (Pharmacia). Gi was separated from Go on a 20-ml DEAE Sephacel column and was eluted with 0.1% lubrol-TED (Tris chloride-EDTA-Dithiothreitol) buffer as described previously [6,21]. Q-Sepharose chromatography greatly increased the resolution of G proteins from other irrelevant proteins. Protein purity was 95% as assessed by immunoblotting using Gi2 α -specific anti-peptide antibodies as well as SDS-PAGE followed by silver staining. Nucleotide binding to purified protein preparations revealed that 0.90 ± 0.2 moles of GTP γ - ^{35}S bound per mole of protein as assessed by the BCA assay (Pierce, Rockford, Illinois). Gi2 α specific anti-peptide antibodies, as well as Gs α , Go α (subtypes 1 and 2), and Gi α common antibodies were a kind gift of Dr. David Manning (U. of Pennsylvania).

Reconstitution of FPR With Gi:

Neutrophil plasma membranes were prepared from degranulated cells as described previously [20]. Photoaffinity agonist labeling and octyl glucoside (octyl- β -D-glucopyranoside or OG) extraction of FPR were done essentially as described [6,18]. Linear sucrose density gradients containing 1% octyl glucoside

(700 μ l, 5-20%) and other variable components to be tested were prepared by pouring step gradients (5, 10, 15, and 20% sucrose) and allowing the gradients to diffuse for 9-10 hours at 4° C to form linear gradients. The octyl glucoside extract was treated with 20 μ M GTP γ S and a G protein and GTP γ S free 4S FPR form was obtained as described previously [6]. Detergent extracts containing the 4S form of FPR were divided into 20 μ l aliquots and purified Gi2 was added to a final concentration of 250-430 nM. The concentration of FPR in the incubation mixtures was estimated to be about 50 to 70 nM assuming 50 to 70% recovery of FPR from whole cells in these crude extracts. Experiments that were sensitive to the FPR concentration supported these estimates of FPR concentration as will be discussed. The FPR-Gi2 mixtures were incubated for 1-2 hours on ice. Longer incubations with Gi2 resulted in virtually identical results. The incubation mixtures were layered on top of the sucrose gradients and centrifuged in a SW55 Beckman swinging-bucket rotor for 10 hours at 45,000 rpm at 4° C. Sedimentation was calibrated with protein standards by centrifuging a mixture of 10 μ g each of cytochrome c (2.1 S), bovine serum albumin (4.4 S), porcine immunoglobulin G (7.7 S), and bovine catalase (11.2 S) in parallel with the experimental gradients. Receptor peaks were localized after fractionation of gradients manually into 55 μ l fractions and individual fractions were subjected to SDS-PAGE. The receptor content was determined by the receptor-specific covalently-bound agonist radioactivity using phosphor technology [23], using a Molecular Dynamics

(Sunnyvale, CA) 400E Phosphor Imager and Image Quant software. Because some of the conditions used altered the sedimentation rate of the receptor, the unassociated, GTP-insensitive form of FPR will be called the slow form while the G protein associated, GTP γ S-sensitive form will be called the fast form.

Mg²⁺ Effects on 7S Reconstitution:

Mg²⁺ concentrations were established using 1 mM EDTA and magnesium chloride in excess of EDTA to the final concentrations indicated in the incubation mixtures during reconstitution. Matching concentration of the divalent ion buffers were included in the sucrose gradients.

Effect of Detergent and FPR Concentration on 7S Reconstitution:

FPR-containing detergent extracts were concentrated twenty fold in Centricon-30 microconcentrators (Amicon, Beverly, MA) and diluted appropriately to obtain different receptor concentrations. For the detergent concentration experiments, the FPR concentrate was diluted into buffers containing appropriate OG concentration to provide similar FPR levels in each of the incubation mixtures. Sucrose gradients were prepared with OG concentrations matching the incubation mixtures.

Effect of Ionic Strength on 7S Reconstitution:

Concentrated FPR was diluted into buffers containing different amounts of NaCl and the reconstitution performed as described above. Appropriate concentrations of NaCl were included in the gradients.

Effect of H⁺ Concentration on 7S Reconstitution:

Aliquots of stock FPR extracts in 10 mM Hepes, 100 mM KCl, 10 mM NaCl, 1 mM EDTA, and 1% octyl glucoside, and the detergent-containing sucrose gradients were adjusted to the pH values indicated in the figure legends using HCl or NaOH.

Effect of Nucleotides on 7S Reconstitution:

10-100 mM guanine or adenine nucleotide stock solutions were prepared in water and adjusted to pH 7.5. Appropriate concentrations of individual nucleotides were added to the FPR-Gi2 complexes and incubated for 0.5-1 hour before layering on the sucrose gradients. Nucleotides at appropriate matching concentrations were included in the gradients. Any other changes in the incubations and/or experimental conditions were discussed in the results section or in the figure legends.

Results

We investigated the formation of molecular complexes between FPR and the Gi2 protein in order to understand the biochemical variables that influence complex formation and to determine whether optimum conditions overlap physiologically relevant conditions. Separation of FPR-Gi2 complexes from uncomplexed FPR was carried out by velocity sedimentation of photoaffinity-radioiodinated FPR in octyl glucoside containing sucrose gradients in the presence and absence of bovine brain Gi2 under a variety of conditions. Radioautographic identification of the agonist-occupied FPR was accomplished by fractionating the sucrose density gradients, running the fractions on SDS-PAGE and exposing dried SDS-polyacrylamide gels to storage screens which could then be developed by phosphor image technology [23]. Figure 7 shows the sedimentation profiles of photoaffinity-labeled, agonist-occupied FPR at four different pH conditions in the presence and absence of Gi2. The slowly-sedimenting form (slow) of FPR, obtained from GTP γ S treated neutrophil membranes, represents G protein-free FPR and sediments in a symmetric fashion with a peak at fraction 4 between pH 6 and 9 as shown in Figure 7 panels B, C, and D. Its normal sedimentation rate is approximately 4S. The sedimentation rate of FPR is increased to an equivalent of 7S in the presence of exogenous bovine brain Gi2 between pH 7 and 9 (Figure 7, C' and D'). The GTP γ S-sensitive, faster sedimenting 7S form (fast) represents a physical complex between FPR and Gi2 as shown previously [18] and discussed further below.



Figure 7. Effect of pH on the sedimentation of FPR and FPR-Gi2 complexes. Octyl glucoside-solubilized, photoaffinity agonist-labeled FPR was incubated without (A-D) or with 400 nM of bovine brain Gi2 (A'-D') to form a fast FPR-Gi2 complex as described in Materials and Methods at pH 5, 6, 7, and 9 respectively. The mixtures were then separated by subjecting them to ultracentrifugation in detergent-containing sucrose density gradient sedimentation for 10 hours at 192,000 g_{av} followed by fractionation into 13 equal fractions. Individual fractions from sucrose density gradients were solubilized in SDS and run on 10% SDS-polyacrylamide gels. The gels were dried and exposed overnight to a storage phosphor screen to obtain radioautographs as described previously [6,23]. FPR is observed as a broad species between the 45- and 68 kD markers (not shown). In order to facilitate comparison of the sedimentation profile of FPR under different conditions, the FPR containing regions on the autoradiographs were excised in the software and stacked sequentially. The detergent containing sucrose solutions were adjusted to matching pH before layering them as step gradients. Between pH 7 and 9 the sedimentation rate of FPR obtained from GTP γ S treated neutrophil membranes is equivalent to that of a globular 4S species such as bovine serum albumin with a peak at fraction number 4. A symmetric two fraction shift into the gradient between pH 7 and 9 is considered as complete complexation of FPR with added G protein as discussed in the text.

Treatment of neutrophil membranes, under conditions known to solubilize many peripheral membrane proteins (1 M NaCl or 5 mM EDTA) in the absence of guanine nucleotides did not result in dissociation of G proteins from the agonist-occupied FPR, indicating tight coupling between the two proteins. The slow FPR form was obtained only after treatment of labeled membranes with 10 μ M GTP γ S.

The effects of pH on the sedimentation profiles of the slow and fast forms can be seen more clearly in Figures 2A and 2B respectively. A stable FPR-Gi2 reconstitution with essentially identical sedimentation profiles was achieved between pH 7 and 9. At pH 5 the receptor appeared in the pellet fraction and was spread across the gradient, suggesting that denaturation as well as aggregation might have occurred at low pH. At pH 5 the receptor content in the pellet fraction increased by about 35% in the presence of added Gi2 (Figure 8b). At pH 6 as well as at pH 11 (not shown) the slow and fast forms of FPR sedimented at distinct locations in the gradients but with peaks at fractions four and five respectively. At these non-optimum pH values the slow and fast forms were separated by one fraction as opposed to a two fraction shift at optimum pH value (pH 7-9). At pH 6 the reconstitution begins to degenerate showing only one fraction shift attributable perhaps to structural perturbations induced at FPR's isoelectric point (6.0 and 6.5) [24]. The sedimentation profile of the slow form of the receptor was somewhat dependent on pH with some indication of aggregation as pH was increased from 6 to 8. Table 2 indicates that at alkaline pH there is a minor

broadening of the sedimentation profiles.

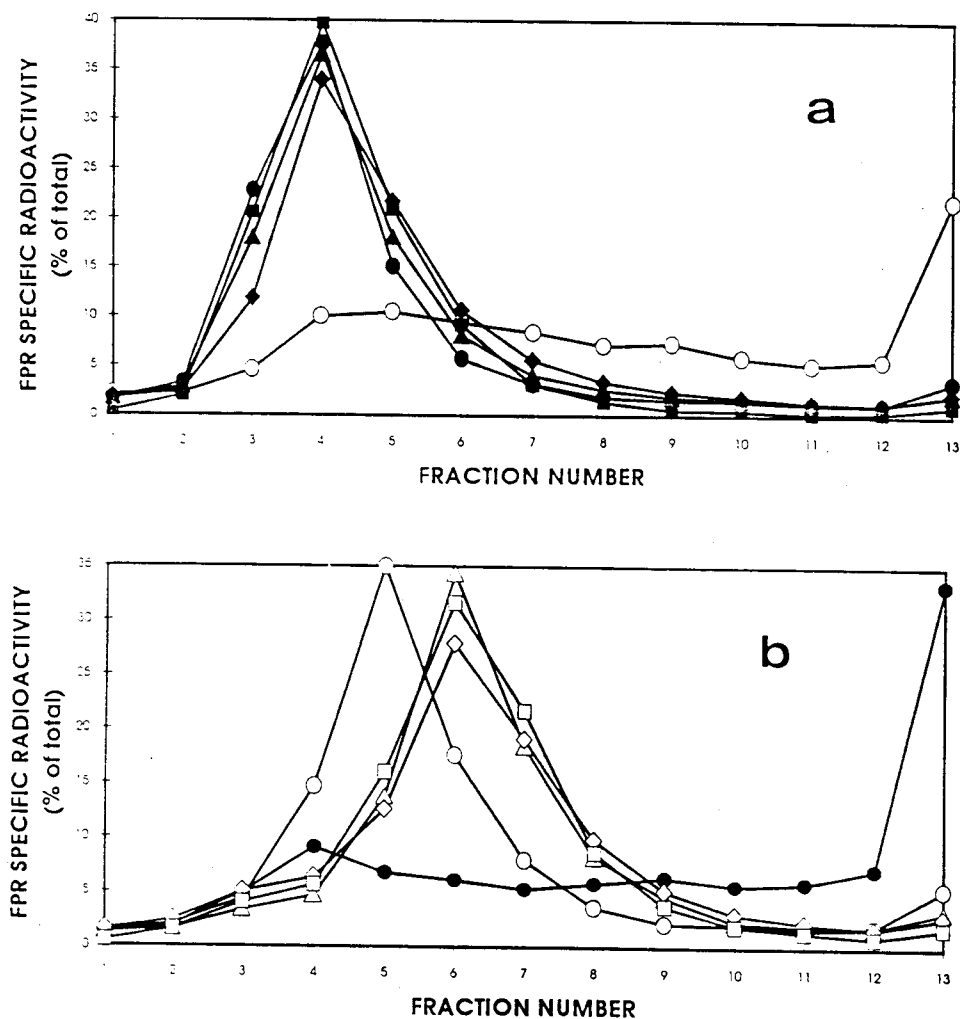


Figure 8. Effect of pH on the reconstitution of FPR-Gi2 complex formation. The receptor content of each fraction, evaluated by SDS-PAGE followed by densitometric methods as described in the legend for Figure 7, is plotted as a function of fraction number which parallels increasing sucrose concentration as described previously [6,18]. Sedimentation profiles shown are: a) effect of pH on the sedimentation of FPR without added Gi2, pH 5 (○), pH 6 (●), pH 7 (▲), pH 8 (◆), and pH 9 (■); b) effect of pH on the sedimentation of the fast form of FPR, pH 5 (●), pH 6 (○), pH 7 (△), pH 8 (◇), and pH 9 (□). Data points represent a single determination typical of three different experiments.

pH	SLOW	FAST
5	-	-
6	17.1	15.6
7	16.3	15.1
8	19.0	20.5
9	17.0	20.0

Table 2. Effect of pH on the half band widths of the sedimentation profiles of slow and fast forms of FPR. The broadness of sedimentation profiles was expressed as the width of each curve at its half maximum height as a percentage of the total width of the gradient such as shown in Figure 8.

Magnesium ions have been shown to have a significant effect on GTP-dependent, agonist binding to FPR [25]. However, as shown in Figure 9, addition of Mg^{2+} to the reconstitution protein mixtures did not substantially alter the sedimentation of either the slow or the fast FPR species. The receptor distribution in the gradient became somewhat narrower with the addition of 100 μM Mg^{2+} (Table 3). A further increase in Mg^{2+} ion concentration (up to 10 mM) had no effect on the receptor sedimentation profile. 1 mM EDTA was used to sequester any traces of magnesium that might have been present in the experiments with zero added magnesium.

In an earlier publication we reported that the reconstitution of the 7S form of FPR is dependent on the concentration of Gi2 with an EC_{50} for the 4S to 7S

conversion of approximately 170 nM [18]. Figure 10 shows the reconstitution of 7S FPR form as a function of FPR concentration. Detergent extracts that are a tenth as concentrated with FPR as control extracts but with the same amount of Gi2, reconstituted the 7S complex completely. The half band width of both the 4S and that of 7S were identical, 17.8% (4S) and 19.7% (7S) at the lower FPR concentration vs. control FPR concentration 17.0% and 19.1% respectively. When the FPR extract was concentrated tenfold no significant FPR was observed in the pellet although the half band width of the slow FPR form increased from 17% to 26% and approximately 50% of the receptors complexed with the added Gi. The 50% complexation of FPR is expected under these conditions given the measured EC_{50} [18] and our estimates of FPR concentration in the incubation mixtures. If the FPR concentration in the control experiments is 50-70 nM as we have estimated then the FPR concentration in ten-fold concentrated extract is 500-700 nM. These concentrations imply that at the EC_{50} reported previously [18] the free Gi2 concentration is 135-145 nM (170 nM minus 25-35 nM bound in FPR-Gi2 complexes) and this would be equal to k_d derived, $\approx 135-145$ nM. The EC_{50} for receptor is 500-700 nM at 430 nM Gi2 (Figure 10) and this implies that the free Gi2 is 80-180 nM in good agreement with the k_d estimated from previous data [18]. In the presence of excess Gi2 virtually all receptors were converted to the fast form indicating that the amount of 7S reconstitution observed at standard FPR concentrations or below was limited by the FPR concentration (data not shown).

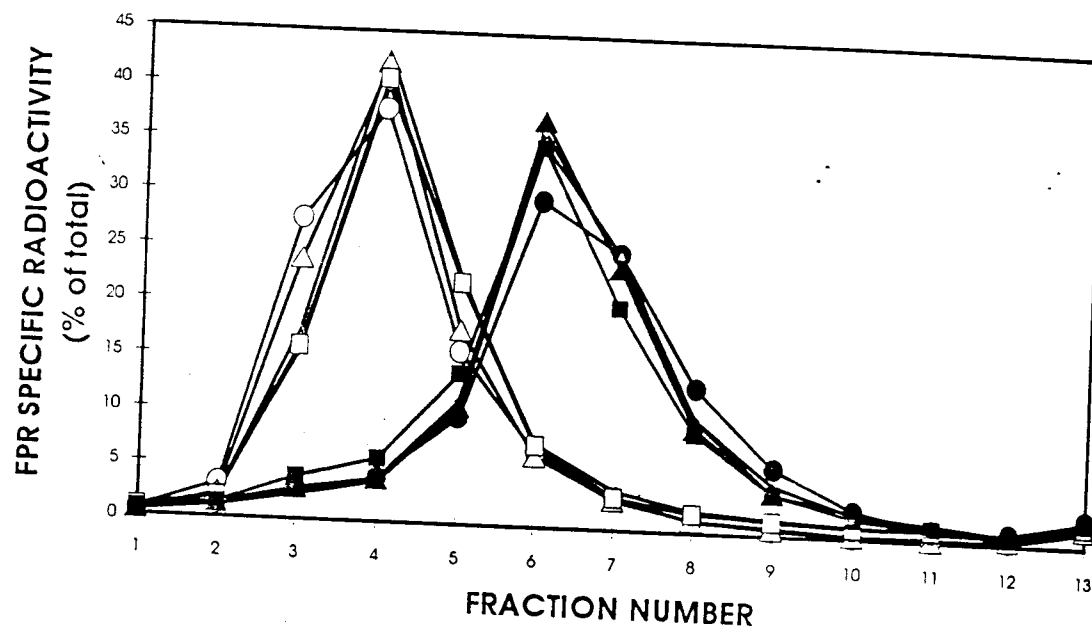


Figure 9. Effect of Mg^{2+} on the reconstitution of FPR-Gi2 complex. Reconstitutions were performed as described in the Materials and Methods section. The plots were obtained using the analysis as outlined in Figure legends 1 and 2. Sedimentation profiles of control, slow (FPR only, open symbols) and fast (with 400 nM Gi2, filled symbols) forms of FPR in the presence of 1 mM EDTA and with no added Mg^{2+} (O, ●). 100 μ M $MgCl_2$ (Δ , $\blacktriangle$), 1 mM $MgCl_2$ (small Δ , $\blacktriangle$), 3 mM $MgCl_2$ (small O, $\bullet$), and 10 mM $MgCl_2$ ($\square$, $\blacksquare$). Data points represent the mean of two independent experiments with average standard errors of less than 8%.

[Mg ²⁺]	SLOW	FAST
0	18.4	21.3
100 μ m	16.5	17.9
1 mM	16.0	18.4
3 mM	16.5	17.2
10 mM	16.0	17.2

Table 3: Percent half band widths of the slow and fast sedimentation forms of FPR in the presence of Mg²⁺. Calculations were done as explained in the legend for Table 2.

[NaCl]	SLOW	FAST
<1 mM	19.2	16.7
10 mM	18.5	21.6
1 M	23.2	30.8
3 M	broad	broad

Table 4. Effect of salt concentration on the percent half band widths (defined in Table 2 legend) of the sedimentation profiles the slow and fast forms of FPR.

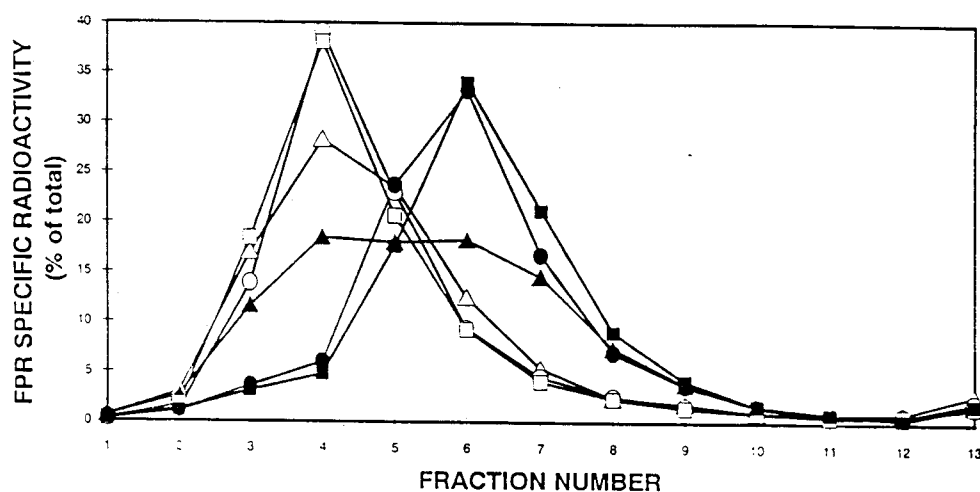


Figure 10: Effect of FPR concentration on the FPR-Gi2 reconstitution. Reconstitution experiments were performed as described in the Materials and Methods in the presence (closed symbols) and absence (open symbols) of 430 nM bovine brain Gi2. Before reconstituting with Gi2, FPR-containing detergent extracts were concentrated tenfold (Δ , $\blacktriangle$) or diluted tenfold ($\square$, $\blacksquare$), with respect to control receptor concentration (O , $\bullet$) and the complexes were analyzed as described in the legend for Figure 7. Data points represent the mean of two independent measurements with average standard errors between the two measurements of less than 9%.

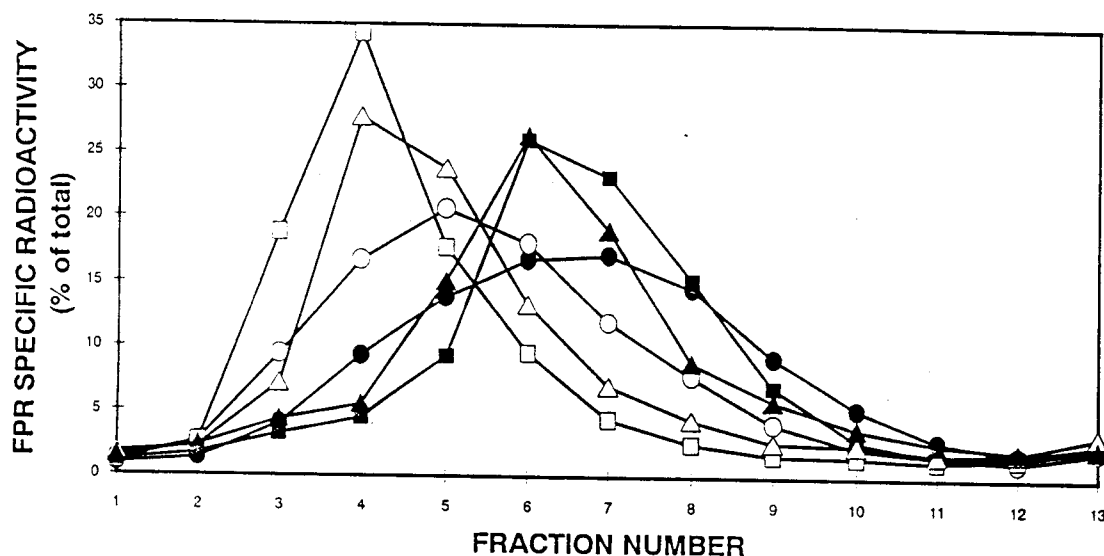


Figure 11: Effect of octyl glucoside concentration on the reconstitution of FPR - Gi2 complexes. A tenfold concentrated FPR extract in 1% octyl glucoside (OG) was diluted into buffers containing different amounts of OG and subsequently incubated with Gi2. Sedimentation profiles of FPR without added Gi2 (open symbols) and FPR with added Gi2 (closed symbols) were plotted as a function of fraction number as described in the legend for Figure 8. Conditions shown are 1% OG w/v (control) (□, ■), 10% OG (○, ●), and 0.1% OG (below CMC) (Δ, ▲). The OG concentration in the sucrose gradients was adjusted to match the incubation conditions. The CMC of OG (at the ionic strength used) is 25 mM or 0.73%. Data points represent a single determination, although similar results were obtained when experiments were repeated.

In order to investigate the nature of the FPR-Gi2 interaction, the dependence of FPR-Gi2 complex formation on detergent concentration and ionic strength was studied. A 1% octyl glucoside (OG) concentration (critical micellar concentration, CMC=0.73%) was used previously to extract functional FPR [17] as well as in reconstitution experiments [6,18]. Figure 11 shows the effects of OG concentration on the complex formation. Although, FPR formed a complex with Gi2 at 0.1% detergent concentration (well below the CMC), the sedimentation profiles were broader for the slow form (21.7% vs. 17.8%) and slightly narrower for the fast form (20.8% vs. 25%). Moreover, at 0.1% OG, the slow and fast forms sedimented closer in the gradient than the two fraction separation obtained at 1% OG. Reconstitutions performed at 1.5% OG were similar to that of control (1% OG) experiments (not shown). At 10% OG concentration the sedimentation profiles for both the 4S and the 7S were almost twice as broad, 35% and 41% respectively and the reconstitution appeared to be incomplete.

The sedimentation profile of fast FPR from reconstitution at low ionic strength ($< 1 \text{ mM Na}^+$) was very similar to the profile at medium to high ionic strengths (100-200 mM Na^+). Figure 12 shows the effect of NaCl concentration on the sedimentation of the FPR species. When reconstitution was performed using 1% OG in double deionized water (no added salt), the profiles were similar to that of control experiments (at 10 mM NaCl and 100 mM KCl). The sedimentation of the fast species in sucrose gradients prepared in distilled water as somewhat

narrower than the slow and the control 7S species (Table 4). Similar results were obtained up to a salt concentration of 200 mM. No significant reconstitution was observed at 1 M salt concentration, where the FPR sedimentation has a broad peak between fractions 4 and 5 both in the presence and absence of added Gi2. At much higher salt concentration (~3 M) receptor appears to form aggregates with large sedimentation rates.

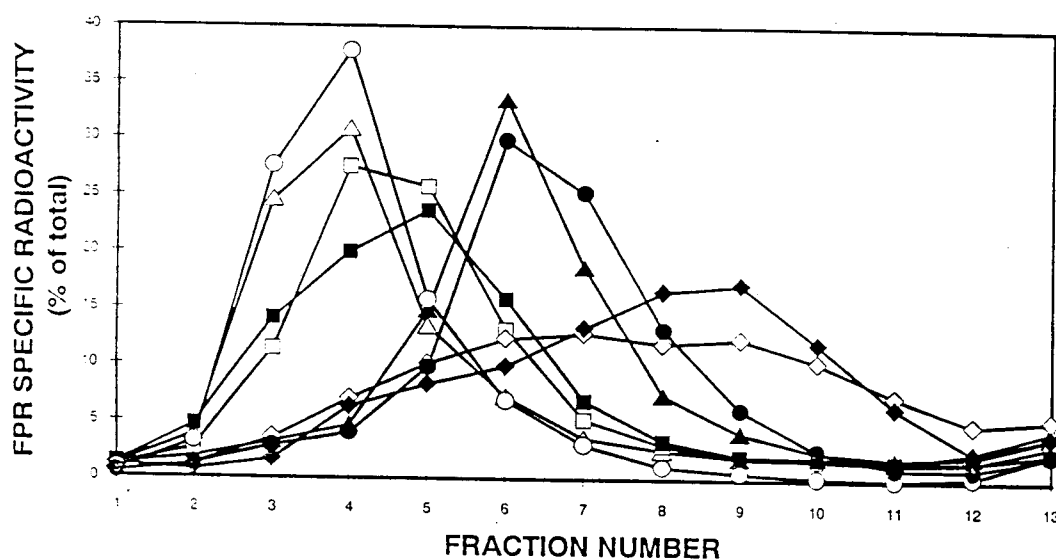


Figure 12: Effect of ionic strength on FPR-Gi2 reconstitution. Concentrated detergent extract containing FPR was diluted into buffers containing different amounts NaCl, and then incubated with Gi2. Sedimentation profiles of FPR without added Gi2 (open symbols) and FPR with added Gi2 (closed symbols) are shown plotted as a function of fraction number. Control conditions in buffer containing 10 mM NaCl (○, ●), buffers made in double deionized water (△, ▲), buffers containing 1 M NaCl (□, ■), and in buffers containing 3 M NaCl (◇ and ◆). The sucrose gradients contained matching amounts of NaCl. Data points represent the mean of two independent experiments and the standard error between the two measurements was less than 7%.

The reconstituted FPR-Gi2 complex can be specifically dissociated by guanine nucleotides. The concentration dependence of the dissociation of the FPR-Gi2 complex by the non-hydrolyzable GTP analogue GTP γ S is shown in Figure 13. At 30 nM GTP γ S, virtually all receptors sedimented as 7S species. More than 90% of the 7S species were disrupted at 100 μ M GTP γ S concentration. GTP γ S had no effect on the sedimentation of 4S species, indicating absence of any dissociable G proteins bound to the agonist labeled receptor. Quantitative comparison of the ability of GTP γ S, GDP β S, GMP and ATP γ S to dissociate 7S is shown in Figure 14. Disruption of fifty percent (IC₅₀) of the 7S complexes by GTP γ S occurred at a concentration of approximately 3 μ M. Magnesium ions (5-10 mM) increased the potency of GTP γ S by a factor of five, as shown by displacement of the FPR-Gi2 dissociation curve to the left. On the other hand, GTP, the natural substrate for G proteins was slightly less effective compared to its non-hydrolyzable analogue GTP γ S, exhibiting an IC₅₀ of 30 μ M (not shown).

Though less potent, GDP β S was also able to dissociate the 7S complex (IC₅₀ 250 μ M), which indicates that the guanine nucleotide binding site on Gi2 α is vacant in the complex with the ligand-occupied FPR. Synergistic effects of Mg²⁺ were less pronounced with GTP or GDP β S (not shown). At a high concentration of 3 mM, GMP was able to dissociate only about 10% of the 7S species. As expected, none of the adenine nucleotides had a significant effect on the FPR-Gi2 complex. ATP γ S at a concentration of 3 mM dissociated about 10% of the FPR-

Gi2 complexes. Mg^{2+} did not increase the efficacy of adenine nucleotides or the non-hydrolyzable adenine analogues.

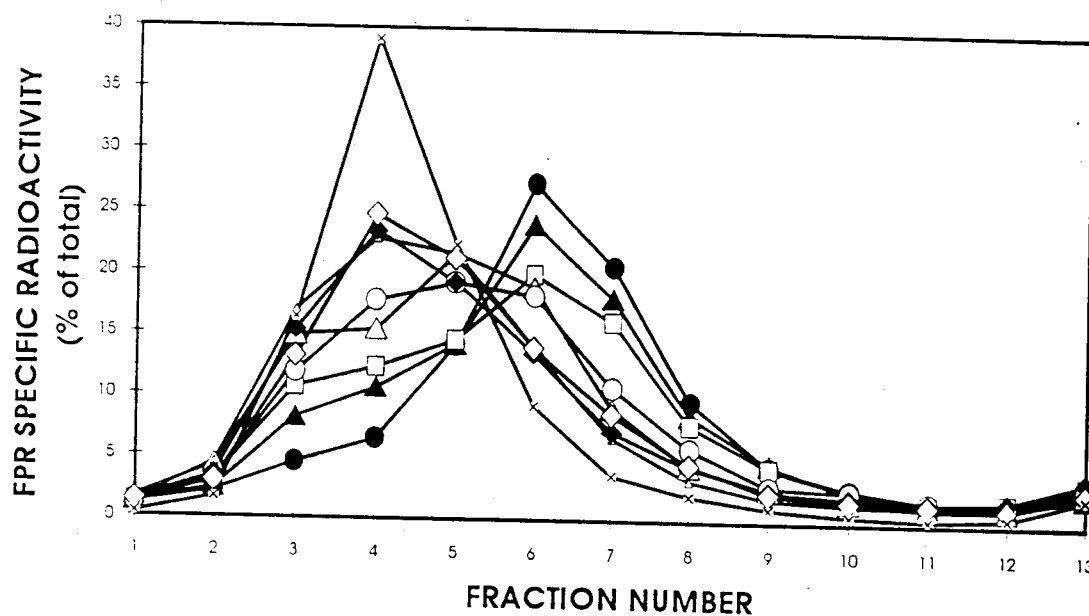


Figure 13: GTP γ S induced dissociation of reconstituted FPR-Gi2 7S complex. The 7S FPR-Gi2 complexes were reconstituted as described in Materials and Methods and incubated further with varying amounts of GTP γ S (in the absence of Mg^{2+}) as indicated; 30 nM (●), 100 nM (▲), 300 nM (□), 1 μ M (large Δ), 10 μ M (O), 100 μ M (small Δ), 1 mM (◆), and 3 mM (◇). The sedimentation profile of 4S species was shown by (X) and for clarity the profile of 7S species was omitted but was identical to that containing 30 nM GTP γ S (●). Data points represent the mean of three independent experiments with standard errors less than 7%.

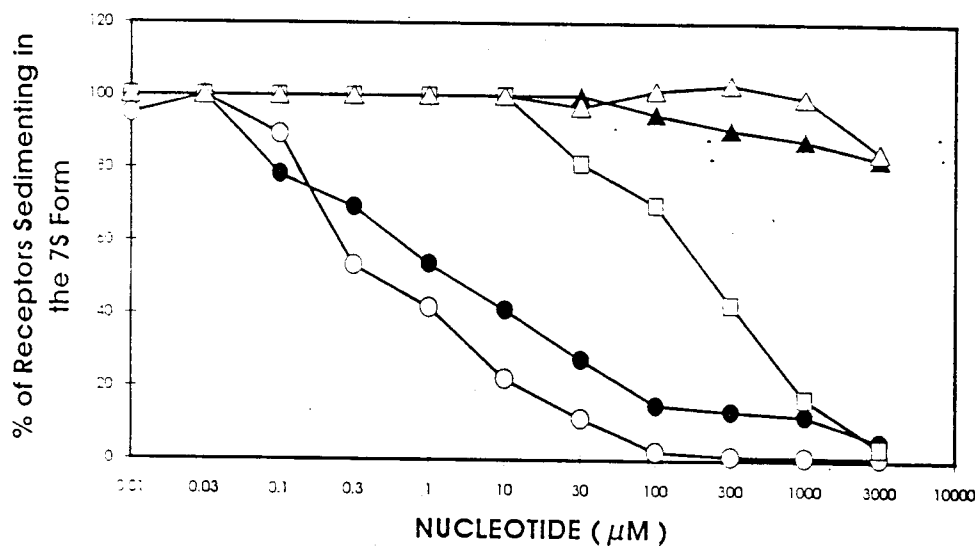


Figure 14: Comparison of the ability of guanine and adenine nucleotides to dissociate the reconstituted FPR-Gi2 complexes. Preformed complexes of FPR-Gi2 complexes were incubated with indicated nucleotides at various concentrations and were analyzed as described in the Materials and Methods section and in the legend for Figure 7 and 8. Dissociation of the 7S complex as a function of nucleotide concentration was plotted to compare the potency of GTP γ S (●), GTP γ S+10 mM Mg²⁺ (○), GDP β S (□), GMP (▲), and ATP γ S (Δ). 100% corresponds to undissociated 7S complex. Data points represent the mean of two independent experiments, except for (GTP γ S) for which three experiments were performed. Standard errors ranged between 7% (GTP γ S) to 12 % for ATP γ S. Results obtained for ADP β S and AMP were similar to that of ATP γ S.

Discussion

The objective of this study was to understand conditions that are important for the interaction between detergent solubilized FPR and Gi2 protein. This study was intended to determine if, apart from detergent, the FPR-Gi2 reconstitution occurred under physiologically relevant conditions. The present work supports the use of the FPR-Gi2 reconstitution system in OG to further analyze the molecular interactions of N-formyl peptide chemoattractant receptor and G protein in the native membrane [6,19].

Guanine nucleotides and their non hydrolyzable derivatives have been shown to modulate high affinity fMLF binding to neutrophil membranes [26]. In this report the specificity of FPR-Gi2 interaction was examined by comparing the ability of different guanine nucleotides and adenine nucleotides to promote dissociation of the reconstituted 7S complex (Figure 14). The non-hydrolyzable GTP analogue GTP γ S (IC_{50} =3 μ M), was most potent, followed by GTP (IC_{50} =30 μ M), and then GDP β S (IC_{50} =250 μ M). Presumably the lower effectiveness of GTP than GTP γ S was due to hydrolysis of GTP. The effect of GMP on the disruption of the 7S form was minimal and adenine nucleotides were without detectable effect. High magnesium ion (at 5-10 mM) increased the potency of GTP γ S in dissociating 7S complexes by a factor of five. These results, along with the functional studies involving GTP γ S induced modulation of ligand binding to FPR [25-27], support the concept that the high affinity fMLF binding is mediated by the

FPR-Gi2 complex and that the conversion of FPR to low affinity state (for agonist) involves a physical uncoupling of FPR from its signal transduction partner Gi2 protein.

The ability of GDP β S to dissociate the 7S FPR-Gi2 complex appears to be highly significant since it indicates that the physical coupling of FPR with Gi2 in detergent coincides with the functional coupling between the two proteins. The primary function of an agonist-occupied receptor is to catalyze the release of bound GDP from G protein to create an empty guanine nucleotide site on the α subunit that can react with either GTP or GDP. The fact that GDP β S dissociates the FPR-Gi2 complex clearly indicates that agonist-occupied FPR empties the G α nucleotide binding site. These results, therefore, confirm the role for agonist-occupied FPR induction of the nucleotide exchange on Gi2 α as has been demonstrated for β 2-adrenergic receptor-Gs coupling [28].

Divalent cations have previously been shown to be important for G protein-coupled receptor functions. Magnesium ions are necessary for the coupling of agonist occupied β 2-adrenergic receptors to Gs protein in membranes [29]. Gierschick and coworkers demonstrated that Mg²⁺ at 1-5 mM, stimulated the binding of fMLF to differentiated HL60 cell membranes, as well as markedly increasing the inhibitory effects of GTP γ S on fMLF binding to FPR [25]. The latter study also suggested the existence of two divalent cation sites on the ligand-FPR-G protein ternary complex and speculated that FPR might interact in a

functionally important manner with divalent cations. Our results indicate that, apart from slight narrowing the sedimentation profiles of slow and fast forms of FPR, magnesium ions have no effect on the Gi2 reconstitution up to 10 mM concentration. Moreover, Mg^{2+} did not change the EC_{50} for the Gi2-induced slow to fast conversion of FPR (unpublished observations), suggesting that the divalent ion is not required for the 7S FPR-Gi2 complex formation in detergent. Thus, the role of Mg^{2+} in the FPR-Gi2 interaction in membrane systems appears to be quite different than in the experiments reported here in detergent extracts. In membranes the largely negative charges on the polar interface of the lipid bilayer may affect the binding of negatively charged sites on the G protein to native membrane receptors. Therefore, in membrane bound FPR studied by the other investigators (cited above) Mg^{2+} ions may have been needed to neutralize charge repulsion to allow FPR-G protein interactions. Such interactions may thus be insensitive to Mg^{2+} when the membrane lipid effects are minimized by nonionic detergent solutions of FPR.

The reconstitution of FPR and Gi2 can be carried out at detergent concentrations below and above the critical micellar concentration (CMC). At concentrations higher than 10 times the CMC the reconstitution is perturbed, resulting in FPR that sediments with broad distributions both in the presence and absence of added Gi2. These broad distributions are probably not due to additional detergent binding to FPR or Gi2, because increased detergent to protein ratios

would decrease the sedimentation coefficient due to the increased partial specific volume of FPR-detergent complexes. It is more likely that at high OG concentrations, micellar aggregation occurs which would produce higher molecular weight, heterogeneously sedimenting species. However, the FPR does not appear to denature in high OG since the sedimentation does shift in a GTP γ S dependent manner (not shown). Similar reconstitutions of a guanine nucleotide sensitive FPR-Gi2 7S complexes are also feasible in Triton X-100 (not shown) which has a much lower CMC and a higher aggregation number than OG. Thus, the tolerance of the FPR-Gi2 reconstitution to a range of detergent conditions supports the use of the reconstitution to reflect physiological protein-protein interactions.

In summary, our results clearly show that physical coupling of FPR and Gi2 occurs over a relatively wide range of protein, pH, detergent, and salt concentrations and thus is probably not an artifactual reconstitution dependent on very special conditions. Reconstitution occurs over a wide range of ionic strengths from <1 mM NaCl, up to the physiological range of 100 mM and above (to 200 mM NaCl). At high 1 M salt concentrations reconstitution is inhibited, probably due to interference with protein-protein interactions and possibly due to denaturation or aggregation. At still higher salt concentration (3 M), FPR sedimented deeper into the gradient in the presence of Gi2 as an approximately 11S species. pH had little effect between 7 and 9. Lastly and most importantly, it is now evident that reconstitution of FPR with Gi2 in octyl glucoside preserves the

complex as an intermediate demonstrating agonist induced vacancy of the guanyl nucleotide binding site and proves its functional nature. Therefore, we believe that the molecular nature of functional signal transducing protein complexes can be probed in this detergent-based physical reconstitution assay. We have used the analysis, characterized in the present paper to map regions on the receptor which are required for receptor-G protein complexation [6,19].

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CHAPTER 4

A CARBOXYL-TERMINAL TAIL PEPTIDE OF NEUTROPHIL CHEMOTACTIC RECEPTOR DISRUPTS ITS PHYSICAL COMPLEX WITH G PROTEIN

Introduction

Neutrophils contribute to immune function by their capacity to carry out chemotaxis, lysosomal enzyme secretion and superoxide production [1]. Bacteria and mitochondria initiate protein synthesis with N-formyl methionine whereas eukaryote protein synthesis begins with methionine. N-formyl peptides can act as chemoattractants by binding to specific neutrophil surface receptors which then trigger the activation of superoxide production and other cellular functions [2-4]. A substantial body of evidence suggests that the N_f-formyl peptide chemoattractant receptor (FPR) mediates signal transduction through interaction with a guanyl nucleotide binding protein or G protein [5-8]. The FPR has been cloned and sequenced [3] and the derived amino acid sequence suggests that FPR belongs to a family of receptors called G protein-coupled heptahelical receptors (see [9-11] for

reviews).

The FPR can be identified by photoaffinity labeling with a derivative of N-formyl peptide and a heterobifunctional radioiodinated crosslinker, F-Met-Leu-Phe-N^ε-(2-(p-azido[¹²⁵I]salicyl-amido)ethyl-1,3'-dithiopropionyl)-Lys (fMLFK-[¹²⁵I]ASD, formerly referred to as FMLPL-[¹²⁵I]SASD) [12]. The affinity labeled FPR from human neutrophils behaves as a mono-disperse species of approximately 63 Kda when analyzed by equilibrium sedimentation analysis in Triton X-100 [13]. Similarly, the FPR from differentiated HL60 cells elutes as a 66 Kda species upon gel-filtration [14]. These estimates are consistent with the apparent size of the affinity labeled receptor on reduced SDS-PAGE gels, where the liganded receptor migrates as a broad band between 50-70 kDa [13,15,16]. Upon enzymatic deglycosylation, the receptor migrates as a sharp 34 kDa band [17].

Photoaffinity labeled, octyl glucoside-solubilized FPR exhibits two size forms with apparent sedimentation coefficients of approximately 4S and 7S. The 7S form can be converted to the 4S form by inclusion of GTPγS in the solubilizing buffer. This conversion occurred with a GTPγS EC₅₀ of approximately 20 nM and appeared to correlate with a reduction in sedimentation rate of G_α and βγ subunits [18].

Detergent-solubilized FPR retains the capability for interaction with endogenous G protein(s) upon reconstitution into phospholipid vesicles [19] or with exogenously added G protein(s) in a concentration dependent manner in octyl

glucoside [20]. The reconstituted 7S form is fully sensitive to guanyl nucleotides, and is immunosedimentable by anti-G α antibodies [20]. Silver staining and immuno blotting of sucrose gradient fractions containing the FPR-G protein complexes indicate that the G protein subunits co-sediment with the 7S form of the receptor. These data suggest that the 7S form of the N-formyl chemotactic peptide receptor represents a physical complex with the G protein.

Such hydrodynamic studies have created a unique opportunity to conduct synthetic peptide competition studies of FPR binding to G protein to gain an understanding of the structural features of the chemotactic receptor that determine its interaction with its signal transduction partner. In this study, we probed the interaction of FPR and bovine G protein using (site-specific) synthetic peptides corresponding to predicted hydrophilic intracellular domains of FPR. Our results show that a fifteen amino acid peptide CT₃₂₂³³⁶ (322-RALTEDSTQTSDTAT-336) from the predicted cytoplasmic tail region of the receptor is able to disrupt the physical complex of G protein and the receptor. This result suggests that the C-terminal tail region of the FPR is involved in the physical coupling of FPR to G protein.

Materials and Methods

Buffers, chemical, methods of cell preparation were as previously described [18,21]. Chemicals used for G protein isolation were as described by Bokoch et

al [22].

Peptide Synthesis and Characterization:

Peptides CT_{322}^{336} ($^{322}RALTEDSTQTSDTAT^{336}$), rCT_{336}^{322} ($^{336}TATDSTQTSDETLAR^{322}$), CII_{127}^{140} ($^{127}VLHPVWTQNHRTVS^{140}$), ($^{170}KTGTVACTFNFSPWT^{184}$) and $CIII_{227}^{239}$ ($^{227}KIHKQGLIKSSRP^{239}$), corresponding to the predicted cytoplasmic surface regions of the FPR (Figure 1), were synthesized by the Fmoc method with a Milligen 9050 automated peptide synthesizer. Deprotection and cleavage was carried out using reagent K (Trifluoroacetic acid 97%, phenol 0.5%, H_2O 1%, ethanedithiol 1%, thioanisole 0.5%). Peptide purity was monitored by HPLC using a Vydac reverse phase C-18 column, and by electrospray-mass spectrometry which revealed a single molecular ion peak corresponding to the molecular weight of the peptides. Peptides corresponding to the extracellular receptor loop EII_{170}^{184} , and neutrophil cytochrome b peptide, CYT_{296}^{306} ($^{296}KVVITKVVTHPFKTIE^{306}$) were made as described previously [23]. The cytochrome b peptide (CYT_{296}^{306}) was a kind gift of Dr. Mark T. Quinn. Peptide stock solutions were made at 50-100 mM in the extraction buffer containing sodium azide (0.02%) and the pH was adjusted to 7.4.

Preparation of G Protein:

G_i was purified from bovine brain as previously described [24], and was

separated from G_o by chromatography on a 20 ml DEAE Sephacel column equilibrated with 25 mM Tris-HCl pH 8, 1 mM EDTA, 1 mM NaCl, 0.6 % Lubrol, and eluted with a linear gradient (200 ml) of 0-250 mM NaCl in the same buffer. Purity was 95% or higher as assessed by $GTP\gamma S$ binding and SDS-PAGE followed by silver-staining. The G protein was the kind gift of Dr. Gary M. Bokoch.

Preparation of the 4S Form of FPR:

Plasma membranes, obtained from stimulated human neutrophils by nitrogen cavitation as described previously by Parkos et al [21], were stored at -70°C until use. The membranes were washed with 1 M NaCl (in "relax buffer," see below) and were resuspended in Hanks buffer (pH 8.5) during the labeling step (at about 1×10^8 cell equivalents per ml). The FPRs were specifically labeled with fMLFK- ^{125}I ASD as described by [12,25]. Briefly, the NaCl washed membranes were incubated with 20-30 nM fMLFK- ^{125}I ASD for 30 minutes on ice in the dark, in foil covered plastic tubes. Nonspecific labeling was assessed in the presence of a 100 fold excess unlabeled fMLFK-ASD. The covalent incorporation of the radiolabel was achieved by irradiating at 370 nm for 10 minutes using a Rayonet Ultraviolet Light Reactor as described previously [12].

The labeled membranes were treated with 10-20 μM $GTP\gamma S$ in order to prevent interference from the endogenous G protein during the reconstitution of the 7S form. $GTP\gamma S$ treatment was done at 4°C and for a duration of 5-10 min. The

treated membranes were washed with 1 M NaCl three times to remove free GTP γ S and peripheral proteins. The washed membranes were pelleted (Beckman Ti60 rotor at 45,000 rpm for 45 min.) and resuspended at 1×10^9 cell equivalents/ml (approx 1 mg/ml protein) in 10 mM Hepes pH 7.4, 100 mM KCl, 10 mM NaCl, 1 mM EDTA, 1 mM PMSF, 10 μ g/ml chymostatin (termed "relax buffer") plus 1% 1-octyl-beta-D-glucopyranoside (octyl glucoside). The membranes were then allowed to extract on ice for 1-2 h and insoluble material was removed by centrifugation at 4°C in a Beckman Ti60 rotor at 45,000 rpm for 30 min.

Peptide Effects on Reconstitution of FPR With G_i Protein:

Linear sucrose density gradients (700 μ l, 5-20%) were prepared by pouring step gradients (5, 10, 15 and 20% sucrose) and allowing the gradients to diffuse into a linear gradient for 10-12 hours at 4°C. All sucrose and peptide solutions were made in the extraction buffer (1% octyl glucoside in relax buffer). The detergent extract containing the FPR was divided into 20 μ l aliquots and G_i was added to a final concentration of 450 nM. The estimated concentration of FPR was about 10-15 pM. The mixtures were incubated for 1-2 hours on ice and then mixed with different concentrations of the peptides. One, two, or eight hour incubations (with G_i) were equally effective in formation of the maximal 7S FPR complex. For most experiments, the peptide containing mixtures were further incubated for 2-5 hours on ice before layering on sucrose density gradients.

However one, two or eight hour incubations with peptide were also found to be equally effective in disrupting the complexes. Moreover, identical results were obtained when the receptor peptides were preincubated with G protein before the addition of FPR. This suggests that these experiments were conducted at effectively equilibrium conditions. Appropriate concentrations of the relevant peptides were also included in the gradients to preclude reformation of complex during sedimentation.

The gradients were then centrifuged in a SW55 Beckman swinging bucket rotor for 8 hours at 45,000 rpm at 4°C and receptor peaks were localized after fractionation of gradients into 55 μ l fractions. Sedimentation experiments were calibrated with protein standards by centrifuging a mixture of 10 μ g each of cytochrome c (2.1S), bovine serum albumin (4.4S), Porcine immunoglobulin (7.7S), and bovine catalase (11.2S) in parallel with experimental gradients. Individual fractions were subjected to SDS-PAGE. G protein content was determined by SDS-PAGE and immuno blot analysis confirming a relatively broad distribution [20] including the FPR-containing fractions. The receptor content was determined by the receptor radioactivity using storage phosphor technology and phosphorimage analysis [26] and also by traditional autoradiographic analysis as described previously [20]. The phosphorimage analysis and the quantitation of the receptor bands was done with a Molecular Dynamics (Sunnyvale, CA) 400E Phosphor Imager and software.

Results and Discussion

Site-specific synthetic receptor peptides corresponding to the predicted interfacial contact sites between receptor and G protein have been observed to interfere with both physical and functional coupling of these macromolecules [27-31]. We report here the use of site-specific synthetic peptides to probe the physical coupling of the N-formyl peptide chemoattractant receptor (FPR) with its signal transduction partner G protein in detergent solution. We investigated the ability of three synthetic peptides corresponding to predicted cytoplasmic domains of the FPR to dissociate a reconstituted receptor-G protein complex. Our assay involves measuring peptide induced changes in the rate of sedimentation of the receptor-G protein as analyzed by velocity sedimentation in linear sucrose density gradients [20].

The structural basis for the interaction between receptors and G proteins in other systems has been intensely studied by site-directed mutagenesis, competition studies using site-specific synthetic peptides, and conventional biochemical methods. Site directed mutagenesis studies suggest that the second and third cytoplasmic loops of β -adrenergic receptor and rhodopsin are involved in receptor-G protein coupling (reviewed in [9]). Synthetic peptide competition studies have implied participation of the second cytoplasmic loop, the N-terminal and a C-terminal regions of the third cytoplasmic loop, and regions of the cytoplasmic tail [9,10,29-32].

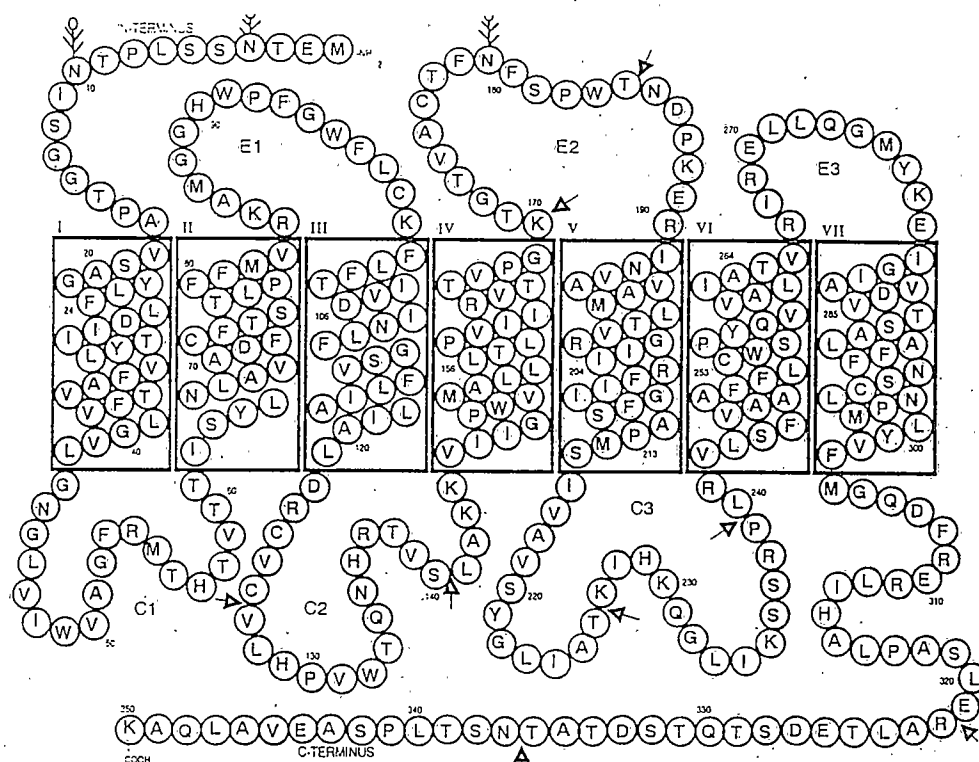


Figure 15. Schematic model of the FPR polypeptide chain in the neutrophil plasma membrane. I through VII represent the putative lipid bilayer spanning domains, which are connected by loops E1, E2, and E3 on the extracellular side and loops C1, C2, and C3 on the cytoplasmic side of the membrane. The asparagine residues at amino acid positions 4, 10, and 179 are predicted to be three potential glycosylation sites on the receptor. Similar seven transmembrane domain models have been proposed and validated to different degrees for a wide range of other integral membrane proteins including bacteriorhodopsin [42,43], rhodopsin [44], and adrenergic receptors [45]. The peptides enclosed by the arrowheads, viz 127-140, 170-184, 227-239, and 322-336, were tested in the reconstitution inhibition experiments.

A schematic representation of the proposed transmembrane topology of FPR is shown in Figure 15. Comparison of sequences of the individual cytoplasmic domains between FPR and other G protein-coupled receptors indicate sequence identities ranging between 7 and 27% (except for the comparison of the first cytoplasmic loop between FPR and rhodopsin which is 46% identical). Such relatively low sequence identities suggest that these regions might confer specificity for receptor-G protein coupling. Selection of FPR peptides for our experiments was based on hydrophilicity [33] and predicted antigenicity [34]. We chose peptide segments CT₃₂₂³³⁶ (³²²RALTEDSTQTSDTAT³³⁶), of the carboxyl terminal tail; CII₁₂₇¹⁴⁰ (¹²⁷VLHPVWTQNHRTVS¹⁴⁰), of the intracellular loop C2; and CIII₂₂₇²³⁹ (²²⁷KIHKQGLIKSSRP²³⁹), of the intracellular loop C3 that are delimited by arrowheads in Figure 15. As control reagents, we also used reverse sequence peptides, peptides with sequences from unrelated proteins CYT₂₉₆³⁰⁶ (²⁹⁶KVVITKVVTHPFKTIE³⁰⁶ of neutrophil cytochrome b heavy chain) and a peptide EII₁₇₀¹⁸⁴ (¹⁷⁰KTGTVACTFNFSPT¹⁸⁴) from the predicted extracellular loop E2 of the receptor.

To probe for interactive sites of the FPR/G protein pair, these peptides were added to the reconstitution mixture of FPR and bovine G_i as described in the materials and methods section. The peptides, at a concentration of 1 mM, were incubated with the receptor-G protein complex and the peptides were present throughout the gradient at the same concentration. However, inclusion of peptides

during the receptor-G protein complexation, or preincubation of the peptides with protein resulted in identical results. Figure 16 shows the effects of various peptides on the rate of sedimentation of the reconstituted FPR. Except for the fifteen amino acid CT_{322}^{336} peptide, none of the other peptides were able to dissociate the reconstituted 7S complex as analyzed on detergent containing sucrose gradients. It is noteworthy that a peptide of identical length but reverse sequence, rCT_{336}^{322} ($^{336}TATDSTQTSDETLAR^{322}$) was also unable to perturb the reconstitution at the same or three fold (3 mM) higher concentration than that used for the native sequence (Figure 3). These results indicate that the peptide-induced dissociation of the 7S receptor-G protein complex was not a result of nonspecific physicochemical reasons. In fact, since the active peptide contains a seven amino acid palindrome constituting a 42% identity with the reverse sequence, the inability of its reverse analog to dissociate the complex confirms the high level of specificity of this competition and localizes the probable active regions of the 15mer to its amino and/or carboxyl termini. Densitometric analysis of the receptor bands on autoradiograms of gels were used to determine the the receptor distribution in the gradient fractions [20]. Figure 17 indicates that the CT_{322}^{336} peptide disrupts the reconstituted 7S complex with an EC_{50} of about 587 μ M. This result suggests that the interaction of this peptide stretch of the FPR and bovine G_i is relatively low affinity but quite comparable to that observed for other receptor peptides [29,30] and G protein peptides [27,35] that dissociate receptor-G protein complexes.

Another common feature of this concentration dependence is the relatively steep inhibition curve with a calculated Hill coefficient of 1.95 [36] corresponding with those calculated with the other systems mentioned above. The interaction between proteins is expected to be multivalent in nature, involving different sites on the interfacial regions. Thus the low affinity observed when a single peptide competes with the full protein-protein interface is not surprising.

Rhodopsin peptides compete with rhodopsin-G protein interactions in the fractional millimolar concentration range [27,29]. The effects of rhodopsin peptides are synergistic when active peptides are used in combination, resulting in lower effective peptide concentrations [27,29]. Unlike in the rhodopsin system, no synergism of FPR-G protein uncoupling was observed when peptides CIII²³⁹₂₂₇, CII¹⁴⁰₁₂₇, and CT³³⁶₃₂₂ were added together (data not shown). This negative result could arise from the fact that peptides used to probe synergism may not have been taken from the correct portion of the cytoplasmic receptor surface. In fact, evidence from the rhodopsin system suggests that two highly conserved amino acids near the amino terminal portion of C-2 loop (glutamic acid and arginine) are involved in functional coupling of the receptor to its G protein [37] but these positions were not included in the peptides tested. FPR like many other G protein coupled receptors contains an aspartic acid and arginine at analogous positions (Figure 15). If another peptide from the regions of the receptor could be found which also displays inhibition of

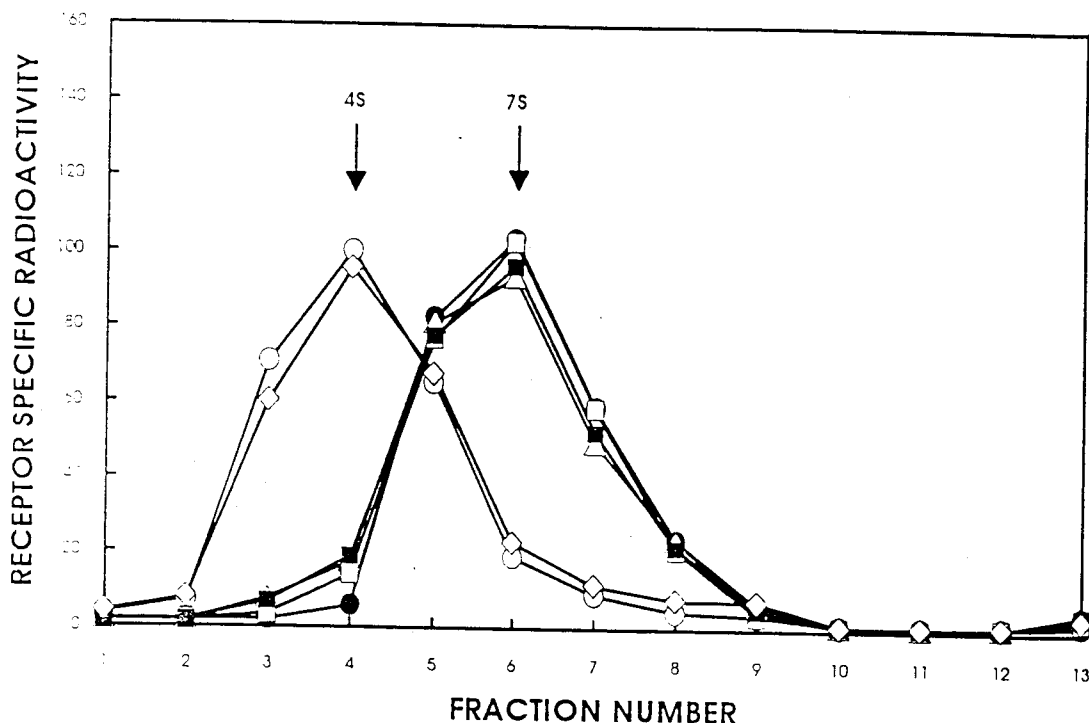


Figure 16. Effect of various FPR peptides on the reconstituted receptor-G protein complexes. Octyl glucoside-extracted, photoaffinity labeled FPR was incubated with 450 nM G_i as described in Materials and Methods. A separation of the complexes on the basis of size was achieved by subjecting the protein mixture to ultracentrifugation in detergent-containing sucrose gradients sedimentation for 8 hours at $192,000 \times g_{av}$ followed by fractionation into 13 equal fractions. The receptor content of each fraction evaluated densitometrically, is plotted as a function of fraction number paralleling increasing sucrose concentration (as described previously [20]). The sedimentation distances in the gradient correspond to 4S and 7S globular proteins and are equivalent to fractions 4 and 6 respectively. Peptides at a concentration of 1 mM were used to assay disruption of the reconstituted 7S complex: CII²³⁹ (²²⁷KIHKQGLIKSSRP²³⁹) (Δ); CII¹⁴⁰ (¹²⁷VLHPVWTQNHRTVS¹⁴⁰) ($\square$); CT³³⁶ (³²²RALTEDSTQTS³³⁶) ($\diamond$); CYT³⁰⁶ (²⁹⁶KVVITKVVTHPFTIE³⁰⁶) ($\blacksquare$); no G_i and no peptide (O); 450 nM G_i and no peptide (●). The sedimentation profile of the receptor-G protein complex in the presence of the rCT³²² (reverse sequence) peptide and the EII¹⁸⁴ peptide was identical to the one that did not contain any peptide (●). Data points represent the mean of two different experiments. Experiments with peptides other than CT³³⁶ ($n=3$) were repeated a third time at a maximum peptide concentration of 3 mM and no disruption of 7S complex was observed.

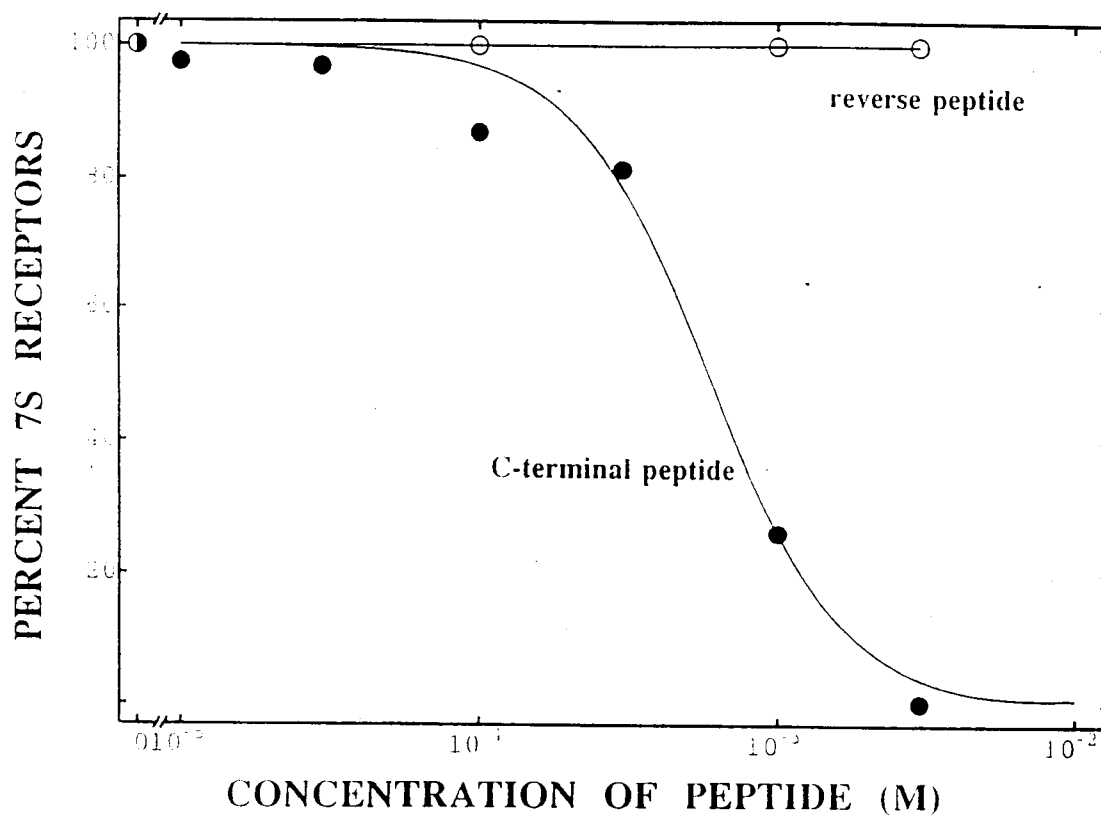


Figure 17. Inhibition of FPR- G_i protein reconstitution as a function of the concentration of CT_{322}^{336} peptide. CT_{322}^{336} (●) and rCT_{336}^{322} (○) peptides were incubated with the reconstituted 7S complex, at concentrations indicated, for 2-4 hours on ice and the complexes analyzed as described in Materials and Methods and in the legend for figure 2. Complete inhibition would be equivalent to pure 4S receptor with no G_i added. Data points represent the mean of three different experiments and the standard deviations were 5.6%, 8.2%, 3.3%, 1.0%, and 8.5% at 10 μ M, 30 μ M, 100 μ M, 300 μ M, and 1 mM peptide concentration respectively. The solid line represents the computer calculated fit of the data to the Hill equation as described by [36].

receptor-G protein coupling it would be interesting to investigate synergistic effects of such a peptide and CT₃₂₂³³⁶.

The fifteen amino acid long CT₃₂₂³³⁶ peptide has 27-40% sequence identity and up to 73% sequence similarity to analogous segments on the cytoplasmic tail regions of various G protein coupled receptors. In addition the alignment of certain amino acids in the sequence appears to be common for several of the other receptors (see Table 5). When the entire carboxyl tail region was compared, however, the amino acid sequence identity ranged between 27% (cAMP-R) and 40% (C5a receptor and rhodopsin). Such identities over the entire tail regions are almost equivalent to functionally unrelated proteins such as chymotrypsin and lysozyme which show a maximum identity of 27 and 20%, respectively. This suggests that the identities over the short stretches may be more relevant to similarities of function of these receptor proteins.

The CT₃₂₂³³⁶ region on FPR is enriched in serine and threonine residues, similar to analogous cytoplasmic tail regions in rhodopsin and β -adrenergic receptors. The serine and threonine residues at positions 334-336 in rhodopsin [38] and numerous sites on the cytoplasmic tail of the β -adrenergic receptor (reviewed in [9]) were shown to be phosphorylated by specific receptor kinases. These phosphorylated regions along with other phosphorylated serine and threonine residues nearby are thought to mediate the binding of the regulatory molecule arrestin to rhodopsin and possibly β -arrestin to the β -receptor [39].

In contrast to earlier reports [40] serine at position 334 (CT 328), two threonine diads at position 319-320 and 335-336 (CT 329) along with an aspartic acid diad at position 330-331 on the carboxyl tail region of rhodopsin have been reported to be important for inhibition of reconstituted GTPase activity using purified rhodopsin and transducin [28]. Moreover, in a recent abstract, experiments were described in which a fusion protein containing the entire carboxyl tail of the FPR and maltose binding protein demonstrated inhibition of high affinity ligand binding to FPR [41]. These results and the significant degree of local sequence identity with rhodopsin support our finding that the CT₃₂₂³³⁶ region on FPR appears to be important for the FPR-G_i coupling.

			PERCENT IDENTITY	PERCENT SIMILARITY	REMARKS
CT 322-336	322- R A L T E D S	T Q T S D T A T -336	-	-	C-Terminus
RHODOPSIN	328- L G D D E A S	I T V S K T E T -342	40	47	C-Terminus
cAMP-R	314- G H P T G D D	V Q Q S S D M E -328	27	47	C-Terminus
hum- β 2-R	375- K L L G E D L	P G T E D F V G -389	33	73	C-Terminus
hum sub P-R	380- L D L T S N C	S S R S D S K T -394	33	60	C-Terminus
hum- α 2-R	289- D A L D L E E	S S S S D H A E -303	33	60	C-III Loop
hum c5a-R	321- N V L T E E S V	V R E S K S F T -336	40	67	C-Terminus

Table 5. Comparison of the CT₃₂₂³³⁶ peptide sequence with other proteins. Sequences were compared by the *BestFit* routine of the Wisconsin GCG software package [46]. *BestFit* makes an optimal alignment of the best segment of similarity between two sequences. Percent similarity takes into account conservative substitutions in the sequences compared. Bestfit uses the "local homology" algorithm of Smith and Waterman (Advances in Applied Mathematics 1981, 2:482-489). The location of the segment compared on various G protein coupled receptors is given in the last column. The peptide segments are manually aligned to show the sequence homology. Highlighted regions represent either amino acid identity or conservative substitutions. The flanking numbers represent the location of the polypeptide segment in the protein. Abbreviations used are CTE 322-336, the CT₃₂₂³³⁶ peptide of the N-formyl chemoattractant peptide receptor; cAMP-R, cyclic AMP receptor from *Dictyostelium Discoideum*; hum- β 2-R, human β ₂-adrenergic receptor; hum sub P-R, human substance P receptor; hum α 2-R, human α ₂-adrenergic receptor; hum C5a-R, human C5a receptor. The shading of the aminoacids was based on the similarity of the residues which are divided into four groups: nonpolar (A,V,L,I,P,M,F,W); polar (G,S,T,C,Y,N,Q); positively charged (K,R,H); and negatively charged (D,E).

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CHAPTER 5

EXTENSIVE CONTACT BETWEEN G_i2 AND N-FORMYL PEPTIDE CHEMOATTRACTANT RECEPTOR OF HUMAN NEUTROPHILS: MAPPING OF BINDING SITES USING SYNTHETIC RECEPTOR-MIMETIC PEPTIDES

Introduction

Neutrophils act as the first line of defense against invading microorganisms in the body. Neutrophils respond to invasions by microorganisms with a complex series of processes (chemotaxis, phagocytosis, lysosomal enzyme secretion and superoxide anion generation) that, with proper function, result in finding, phagocytosis and killing of the invaders. N-formyl peptides act as chemoattractants for neutrophils by binding to specific surface receptors which then trigger the activation of the cellular events mentioned above [1-3]. Since bacteria initiate protein synthesis with N-formylated methionine, the neutrophils may use the N-formyl peptide chemoattractant receptor (FPR) to find sites of infection in tissues and organs. A primary molecular event mediated by FPR is the induction of GDP-

GTP exchange on heterotrimeric G proteins by agonist-occupied (FPR) [4-7].

The FPR cDNA has been cloned and sequenced [2], and hydropathy analysis of the derived amino acid sequence and conserved amino acids [8-10] identifies FPR as a member the family of receptors called G protein-coupled receptors (GPCR) [11]. A striking feature of GPCR is the presence of seven predominantly hydrophobic regions which are thought to form membrane spanning α -helices, connected by relatively hydrophilic polypeptide domains on the extracellular as well as the cytoplasmic side of the receptors. The seven transmembrane helix motif was found by electron diffraction analysis of bacteriorhodopsin, a non-G protein-linked light-driven proton pump [12]. It has been proposed that many of the salient features of the bacteriorhodopsin transmembrane motif are present in rhodopsin [13] and this has been partially validated for rhodopsin [14] and β 2-adrenergic receptor (reviewed in [11]). Based on sequence homology between FPR and other GPCR, working models of the transmembrane organization of FPR have been proposed [2,15,16].

Photoaffinity agonist-labeled FPR from human neutrophils behaves as a monodisperse species of approximately 63 kD when analyzed by equilibrium sedimentation in Triton X-100 [17]. FPR obtained from differentiated HL60 cells and solubilized in sodium cholate elutes as a 66 kD species upon gel filtration [18]. These results are consistent with the apparent size of the photoaffinity agonist-labeled receptor on denaturing sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE) gels, where it migrates as a broad band between 50 and 70 kD [17,19,20]. The non-glycosylated FPR expressed in *E. coli* [21] and enzymatically deglycosylated native FPR [22], migrates as a sharp 35 kD band on SDS-PAGE.

Photoaffinity agonist-labeled, FPR solubilized in octyl glucoside from neutrophil plasma membranes exhibits two size forms with apparent sedimentation coefficients of approximately 4S and 7S which can be resolved by velocity sedimentation in linear sucrose density gradients [23]. The more rapidly sedimenting 7S form can be converted to the 4S form by inclusion of GTP γ S in the solubilization buffer. Detergent-solubilized FPR retains the capacity for interaction with added G protein(s), in a concentration-dependent manner, upon reconstitution into phospholipid vesicles [24] or in detergent extracts [25]. The 7S form can be produced from 4S FPR and Gi2 and is quantitatively dissociated to 4S by guanine nucleotides. It is immunosedimentable by anti-Gi2 α antibodies [25]. These data indicate that the 7S form is a physical complex of FPR with the Gi2 protein.

Study of the contact sites responsible for GPCR and G protein coupling is being actively pursued using a variety of approaches including site-directed mutagenesis [26], construction of chimeric receptors [27], as well as using synthetic peptides to block and map putative interactive regions [8,28-30]. Studies with rhodopsin [8,29,31], muscarinic receptors [32], and adrenergic receptors [27]

suggest that receptor regions involved in G protein coupling include parts of the second intracellular loop, amino and carboxyl portions of the third loop, and the portion of the C-terminus closest to the membrane. The carboxyl and the amino terminal regions of the α subunits of G proteins have been shown to mediate receptor interaction [29,33]. The $\beta\gamma$ subunits of the heterotrimeric G protein also appear to contribute to receptor-G protein specificity [34-36].

Reconstitution of FPR-Gi2 complexes and hydrodynamic analysis has allowed us to conduct synthetic peptide competition studies to map the regions of FPR that mediate binding to Gi2. These studies complement activity-based assays, which cannot discriminate between binding and activation and site-directed mutagenesis which may induce structural perturbations in proteins, and thus can not lead to unequivocal identification of active-site regions. Together, however, they provide a relatively comprehensive way to address structure-function questions in the absence of X-ray crystal structure.

We have previously shown that a fifteen amino acid peptide (CTE) from the middle of the C-terminal tail of FPR was able to dissociate the reconstituted 7S FPR-Gi2 complex [15] while hydrophilic peptides from the putative centers of the second (C2 and C3) cytoplasmic loops were totally without effect. In the current study, we investigated a wider range of the putative cytoplasmic regions of FPR that might be involved in Gi2 binding using synthetic peptide competition. Our results provide evidence that FPR must interact with Gi2 using regions of its

structure that appear to be at or near the membrane cytoplasmic interface and not those calculated to be most hydrophilic in the predicted cytoplasmic loop regions. We conclude that the first and second cytoplasmic loops as well as putative juxtamembrane regions of the second and third loops of FPR serve as recognition sites for heterotrimeric Gi2. These results along with our previous finding of a middle region of the COOH tail peptide (CTE) involvement in the FPR-Gi2 interaction, indicates that the physical coupling between FPR and Gi2 involves extensive contact between the two proteins.

Materials and Methods

Buffers, chemicals, and methods of cell preparation were used and obtained as previously described [23,37]. Chemicals used for G protein isolation were as described by Bokoch et al, [38].

Peptide Synthesis and Characterization:

The peptides shown in the figure legends and control peptides E1A, E2A, and Cyt-b were synthesized and characterized as described previously [15]. The peptides were purified by high-performance liquid chromatography in gradients of acetonitrile/water/2 mM HCl, and the purity of the fractions was assessed by electrospray mass spectrometry in methanol/water/acetic acid (50:50:1) solvent. Peptide stock solutions were 25-100 mM in the extraction buffer with 0.02%

sodium azide and the pH was adjusted to 7.5. A control peptide from the neutrophil cytochrome b heavy chain (gp91^{phox}) was a kind gift of Dr. Mark Quinn.

Preparation of G Protein:

Gi was purified from bovine brain as previously described [39] with the following modifications. For the first two chromatographic steps Q-Sepharose Fast Flow ion exchange media (Pharmacia) and Sephacryl 200-HR gel filtration media (Pharmacia) were used respectively. Gi was separated from Go on a 20-ml DEAE Sephacel column using 0.1% lubrol in TED buffer as described previously [15]. Purity was about 95% as assessed by immunoblotting using Gi2 α -specific anti-peptide antibodies and SDS-PAGE followed by silver staining. Nucleotide binding to purified preparations showed that 0.90 ± 0.2 moles of GTP γ ³⁵S bound per mole of protein as assessed by the BCA protein assay (Pierce, Rockford, Illinois). Gi2 α -specific anti-peptide antibodies as well as Gs α , Go α (subtypes 1 and 2), antibodies were a kind gift of Dr. David Manning (U. of Pennsylvania).

Peptide Effects on Reconstitution of FPR With Gi2:

Preparation of buffers, neutrophil plasma membranes, photoaffinity agonist FPR labeling, octyl glucoside extraction, and peptide incubations were carried out essentially as described previously [15,40,41]. Briefly, octyl glucoside extracts of purified plasma membranes, containing the 4S form of FPR, were divided into

aliquots and Gi was added to a final concentration of 250-430 nM. The concentration of FPR in the incubation mixtures was estimated to be about 50-70 nM assuming 50-70% recovery of FPR from whole cells in the crude extracts. The FPR-Gi2 mixtures were incubated for 1-2 hours on ice and then different concentrations of the peptides were added. The peptide-containing mixtures were further incubated for 1-2 hours on ice before layering on sucrose density gradients. The gradients contained the same concentrations of peptide as were used in the corresponding incubation mixtures to preclude re-formation of complexes during sedimentation. Linear sucrose density gradients (700 μ l, 5-20%) containing 1% octyl glucoside were prepared by pouring step gradients (5, 10, 15, and 20% sucrose) and allowing the gradients to diffuse into a linear gradient for 10 hours at 4° C. Longer incubations with the peptides or preincubating the peptides with Gi2 before addition of FPR resulted in identical results, indicating that these experiments were effectively conducted at equilibrium.

The gradients were centrifuged in a SW55 Beckman swinging-bucket rotor for 10 hours at 45,000 rpm at 4° C and receptor peaks were localized after fractionation of gradients into 55 μ l fractions. Sedimentation experiments were calibrated with protein standards by centrifuging a mixture of 10 μ g each of cytochrome c (2.1 S), bovine serum albumin (4.4 S), porcine immunoglobulin G (7.7 S), and bovine catalase (11.2 S) in parallel with experimental gradients. Individual fractions were subjected to SDS-PAGE. The receptor content was

determined by the receptor-specific agonist radioactivity using phosphor technology and phosphor image analysis [42], with a Molecular Dynamics (Sunnyvale, CA) 400E Phosphor Imager and Image Quant software.

Results

We have previously shown that a fifteen amino acid peptide from the middle of the carboxyl terminal tail of FPR specifically interferes with FPR-Gi2 complexation, while the hydrophilic putative central regions of the second and third cytoplasmic loops were totally ineffective [15]. Here we investigated thirteen additional peptides, distributed over the putative cytoplasmic surface and juxtamembrane regions of FPR for their ability to interfere with FPR-Gi2 interaction. The Initial focus of this study was on the juxtamembrane regions of the third cytoplasmic loop of FPR (viz., C3A and C3B in Figure 20). Analogous regions have been shown to be important for G protein coupling in the case of rhodopsin [43] and the β 2-adrenergic receptor [44,45]. Overlapping peptides covering the third cytoplasmic loop of FPR, including residues from the putative juxtamembrane interface were synthesized (Figure 20) and tested for their ability to block the FPR-Gi2 interaction.

Covalent labeling of FPR with radio-labeled agonist allows us to study FPR complexes without purification of FPR [15,17,25,46]. Our assay involves specific dissociation of the FPR-Gi2 complexes by receptor peptides and separation of

complexed and uncomplexed FPR by ultracentrifugation in detergent-containing linear sucrose density gradients. Quantitation of the radioactive agonist-labeled FPR content in sucrose gradient fractions was obtained by subjecting dried SDS-polyacrylamide gels to radioautography using storage phosphor image analysis [42]. Figure 18 shows the sedimentation profile of agonist-labeled FPR under three different conditions. Panel A in this figure shows the sedimentation profile of the G protein free, 4S form of FPR form obtained from GTP γ S-treated neutrophil membranes [15,25]. Panel B in Figure 18 shows that, in the presence of added exogenous Gi2, there is a two fraction peak shift in the sedimentation of the agonist-labeled FPR, which corresponds to a 7S sedimentation coefficient. Multiple lines of evidence from our earlier report [25] suggest that the faster sedimenting 7S species represents a physical complex of FPR and Gi2.

The 7S species can be specifically disrupted by certain FPR peptides and not by others. Panel C of Figure 18 shows the effect of a peptide corresponding to the carboxyl terminal end of the putative third cytoplasmic loop of FPR that we call C3A. This peptide fully dissociates the reconstituted 7S FPR-Gi2 complex (see Figure 20 and Table 6 for the peptide sequence and its location in a proposed model FPR structure). This particular region on FPR has not previously been shown to mediate FPR-Gi2 interaction. As seen in panel C of Figure 18, the C3A peptide at 3 mM concentration was able to fully shift the 7S FPR sedimentation profile to that of a 4S species indicating that C3A peptide completely dissociated

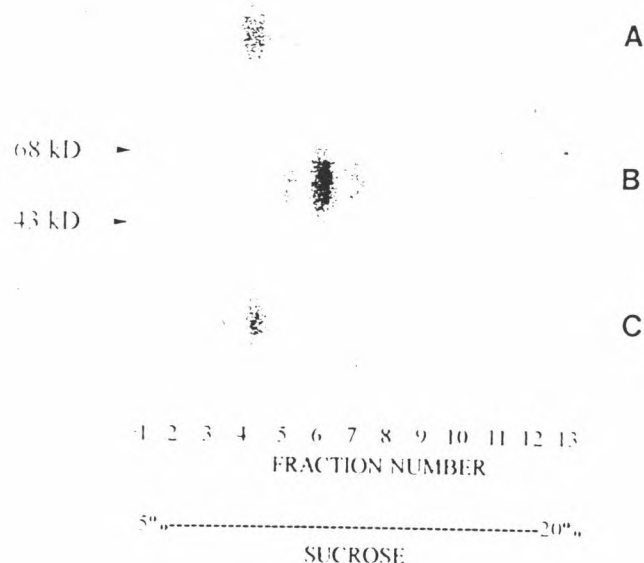


Figure 18. Disruption of stable FPR-Gi2 complexes by a receptor-mimetic peptide from the predicted third cytoplasmic loop of FPR (C3A, see Table 6). Storage phosphor visualization of the ^{125}I photoaffinity labeled and reconstituted FPR-Gi2 complexes separated by velocity sedimentation in linear sucrose density gradients containing 1% octyl glucoside is shown. Fractions from the density gradients were solubilized in SDS and run on 10% SDS-polyacrylamide gels. The gels were dried, FPR visualized and quantitated after a 20 hour exposure to storage phosphor screen [15,42]. FPR was uniquely labeled and is observed as a broad species between the 43- and 68-kDa markers. Panel A shows the FPR sedimentation profile in region of the gel encompassing the FPR molecular weight range. The sedimentation distances of FPR alone and FPR-Gi2 complexes in the gradient correspond to 4S and 7S globular proteins and are equivalent to fractions 4 and 6. Panel B shows the symmetric shift in sedimentation to 7S from 4S that occurs in the presence of added Gi2 (400 nM). Panel C shows disruption of the 7S complex by FPR C3A peptide at 3 mM (see legend for Figure 19 and Table 6 for the C3A peptide sequence). The peptide(s) were incubated with the reconstituted 7S complex, at concentrations indicated, for 2-4 hours on ice prior to sedimentation. Similar shifts in sedimentation of the reconstituted 7S complex were observed with other active FPR peptides.

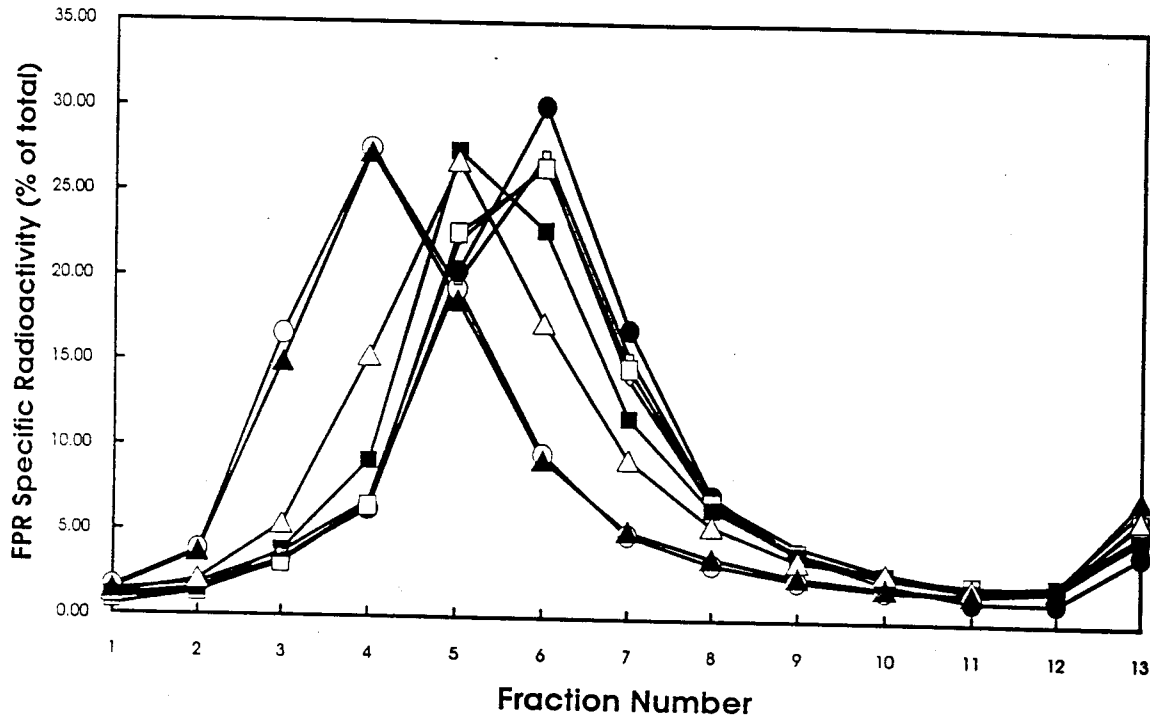


Figure 19. Concentration dependence of C3A-mediated disruption of FPR-Gi2 complexes. Octyl glucoside-solubilized, photoaffinity labeled FPR was reconstituted with exogenously added bovine brain Gi2 to form 7S complex as described in Materials and Methods. The uncomplexed FPR and FPR complexed with Gi2 were resolved by ultracentrifugation in detergent-containing sucrose gradient sedimentation for 10 hours at $192,000g_{av}$ followed by fractionation into 13 equal fractions. The receptor content of each fraction, evaluated densitometrically from radioautographs or by storage phosphor technology such as shown in Figure 18, is plotted as a function of fraction number which parallel increasing sucrose concentration, as described previously [15,25]. The sedimentation profile of FPR alone (4S) and FPR-Gi2 (7S) are shown by (O) and (●) respectively. C3A (230-KQGLIKSSRPLRVLSFV-246) peptide at various concentrations [10 μ M, (small $\square$); 30 μ M, ($\diamond$); 100 μ M, (large $\square$); 300 μ M, ($\blacksquare$); 1 mM, ($\triangle$); and 3 mM, ($\blacktriangle$)] was used to assay disruption of reconstituted 7S complex. In the presence of control peptides (Table 6) from the FPR extracellular face E1A and E2A, neutrophil cytochrome b peptide gp91^{phox}, Gi2 α peptide (Gi2-MKI), or from human cytosolic β -actin (actin-MKI) (not shown), the sedimentation profile was identical to that for the assay that did not contain any peptide (●). Data points represent the mean of three independent experiments and the standard error ranged from 2.7% to 4.7%. Points at 10 and 30 μ M peptide concentration were measured but are obscured by the 100 μ M points on this plot.

PEPTIDE NAME	POSITION AND SEQUENCE	LENGTH	IC ₅₀
C1W	43-GNGLVIWVAGFRMTHVT-61	19	100 μ M
C2A	134-QNHRTVSLAKKVIIGPW-150	17	100 μ M
C2M	127-VLHPVWTQNHRTVS-140	14	inactive
C2B	119-IALDRCVCVLHPVWT-133	15	1 mM
C2W	122-DRCVCVLHPVWTQNHRTVSLAKK-144	23	20 μ M
C2W++	119-IALDRCVCVLHPVWTQNHRTVSLAKKVI-146	28	0.5 mM
revC2W	144-KKALSVTRHNQTWVPHLVCVCRD-122	23	2 mM
C3A	230-KQGLIKSSRLRVLSFV-246	17	1.4 mM
C3M	227-KIHKQGLIKSSRP-239	13	inactive
C3B	210-FSAPMSIVAVSYGLI-224	15	0.5 mM
revC3A	246-VFSLVRLPRSSKILGQK-230	17	>10 mM
CTA	301-YVFMGQDFRERLIH-314	14	insoluble
CTB	304-MGQDFRERLIHALPAS-319	16	>3 mM
CTC	315-ALPASLERALTEDST-329	15	>3 mM
CTD	330-QTSDTATNSTLPSAE-344	15	inactive
CTE	322-RALTEDSTQSDTAT-336	15	400 μ M
CTZ	337-NSTLPSAEVALQAK-350	14	inactive
revCTE	336-TATDSTQTSDETLAR-322	15	inactive
CONTROL PEPTIDES			
FPR-E1A	85-KAMGGHWPFGWFLCK-99	15	inactive
FPR-E2A	170-KTGTVACTFNFPWT-184	15	inactive
gp91-phox	296-KVVITKVVTHPFKIE-306	16	inactive
Gi2-MKI	53-MKIIHEDGYS-62	10	inactive
Actin-MKI	190-MKILTERGYS-199	10	inactive

Table 6. Summary of all peptides used in the present study of peptide induced disruption of reconstituted FPR-Gi2 complexes. Peptides were synthesized and characterized as described in the Materials and Methods section and were derived from the known amino acid sequence [72] and the published topographic model of FPR [15]. The alphanumeric names of FPR peptides denote their disposition in the proposed model, C2 for second cytoplasmic loop, CT for carboxyl terminal tail, E1 for first extracellular loop etc. The middle regions of the predicted loops and entire predicted loop sequences are denoted by the letters "M" or "W" respectively. Control peptides gp91^{phox} was from neutrophil cytochrome b heavy chain, while peptides Gi2-MKI and actin-MKI are from human Gi2 α subunit and human cytosolic β -actin respectively. The numbers flanking the first and last residues of the peptides shown correspond to their location in the intact protein sequence. Peptides were assayed for their ability to dissociate reconstituted FPR-Gi2 7S complexes as described in the Materials and Methods section and in the legends for Figures 1 and 2. Peptide concentration required to dissociate 50% of the 7S complexes (IC₅₀ values) are measured from peptide titration plots such as shown in figures 3 through 6.

the reconstituted 7S FPR-Gi2 complex. The peptide effects studied appeared to be at equilibrium since peptides at the same concentration were included in the analytical gradients and inclusion of peptides during 7S reconstitution or preincubation of the peptides with Gi2 produced identical results. Control peptides having similar amino acid composition to that of C3A but having different sequences and/or lengths eg., neutrophil gp91^{phox}, FPR extracellular domain peptides, peptides derived from the Gi2 α subunit or actin (as shown in Table 6) did not dissociate the 7S complex up to a concentration of 5 mM. Taken together with our published results on the carboxyl terminal tail peptides of FPR [15], the current observations suggest that peptide-induced dissociation of 7S was not a result of nonspecific effects.

The disruption of the FPR-Gi2 complex depends on the concentration of the active peptides in a graded manner. Figure 19 shows the dependence of the sedimentation of agonist-labeled FPR in the presence of Gi2 at several different C3A peptide concentrations. Densitometric scans of radioautographs such as shown in Figure 18 were used to measure the receptor content of each sucrose gradient fraction and the receptor content was plotted as a function of fraction number. The effectiveness of each peptide in dissociating the FPR-Gi2 complex was assessed by plotting the disappearance of radio-labeled FPR in fraction 6 and determining a fifty percent inhibition concentration (IC₅₀) as shown in Figure 20. The relatively narrow distributions for the two FPR forms in the gradients (half-

band width, ~22% of gradient) shown in Figure 19 are comparable to other membrane proteins in detergent-containing sucrose gradients (half-band width, 15-20%) [47,48]. The sequences of all of the peptides used in this work, their name designations and IC_{50} 's are described in the figure legends and are summarized in Table 6.

To examine the role of other regions of the putative third cytoplasmic loop of FPR in FPR-Gi2 interaction, similar experiments were carried out with other peptides. Figure 20 shows a quantitative comparison of various FPR peptides on their ability to interfere with FPR-Gi2 complexation. The disruption of the 7S complexes is plotted as a function of increasing peptide concentration. The IC_{50} 's for C3B and C3A were approximately 0.5 and 1.4 mM. Previous studies involving peptides corresponding to various other GPCR and G proteins reported a similar range of peptide concentrations that were effective in blocking such interactions [29,33,49,50]. The reverse sequence of C3A peptide, (revC3A) displayed less than one tenth the activity of C3A as estimated from its extrapolated IC_{50} value whereas revCTE was inactive. These results are consistent with other observations that reverse sequence peptides (retro all-L peptides) may form topological mirror images of native sequences (normal all-L peptides) and may have biological activity [51].

The putative second cytoplasmic loop of many GPCR has been shown to be involved in receptor-G protein coupling [8]. The amino terminus of the second putative cytoplasmic loop of most G protein-coupled receptors contains an acidic

amino acid followed by a basic amino acid, the D/E,R diad. This diad has been shown to be involved in G protein activation, and reversal of the charge pair from Glu-Arg to Arg-Glu abolished Gt binding to rhodopsin [52]. Mutation of the arginine residue to a leucine or a tryptophan is implicated in human retinitis pigmentosa [8].

We applied our hydrodynamic analysis to examine the role of the putative second cytoplasmic loop of FPR in Gi2 binding using several overlapping peptides as shown in Figure 21. The sequence corresponding to the entire proposed second cytoplasmic loop (C2W) is the most active peptide investigated, with an IC_{50} value of approximately 20 μM . Similar to results on the third loop [15], the middle region peptide (C2M) of the second loop was inactive. Comparing the activity of C2W and C2M suggests that residues 122-125 and/or residues 141-144 may be critical for the activity (Figure 21). However, unlike observations on putative third loop peptide sequences containing larger hydrophobic stretches appeared to be less active than the C2W peptide in our analysis. Peptide C2W++ containing the putative C2 loop as well as additional juxtamembrane residues at both its amino and carboxyl ends was ten fold less effective than C2W. The two flanking "half loop" peptides C2B and C2A exhibit IC_{50} values of 1.5 mM and 200 μM respectively. The revC2W peptide, the reverse analogue of C2W, was 100 fold less active in dissociating the 7S complex than the native sequence.

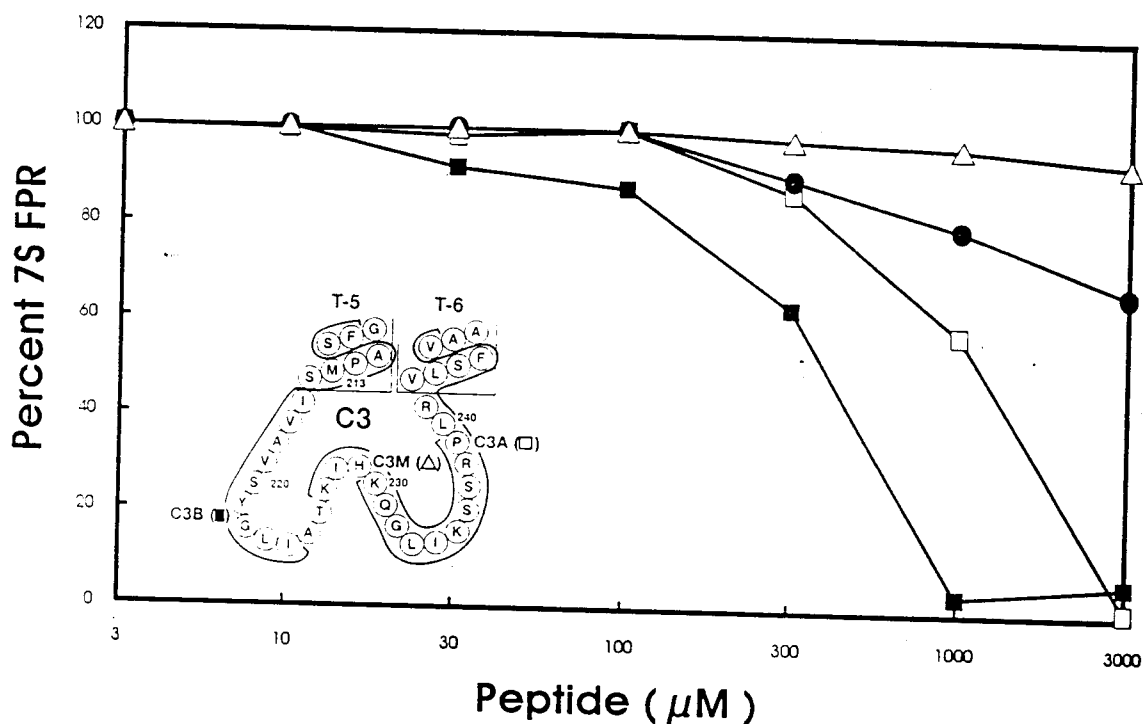


Figure 20. Comparison of the disruption of reconstituted 7S by synthetic peptides from the putative third cytoplasmic loop of FPR. Inset shows a diagram of the third cytoplasmic loop of FPR and showing the location of various peptides tested. T-5 and T-6 represent transmembrane helical regions five and six close to the cytoplasmic face of the membrane. Cytoplasmic C3 loop connects membrane domains T-5 and T-6. Numbers indicate the residue numbers in the linear sequence of FPR. The percent of the total FPR sedimenting with apparent sedimentation coefficient of 7S was determined by sedimentation analysis as described in the legend for Figure 19 for each concentration indicated for several peptides whose sequence were derived or related to that of the C3 loop of FPR. Peptides used were C3A, (230-KQGLIKSSRPLRVLSFV-246) ($\square$); C3B, (210-FSAPMSIVAVSYGLI-224) ($\blacksquare$); revC3A, (246-VFSLVRLPRSSKILGQK-230) ($\bullet$); and C3M, (227-KIHKQGLIKSSRP-239) ($\triangle$). Complete inhibition would be equivalent to pure 4S form of FPR with no Gi added. Data points represent the mean of three different experiments and the standard error ranged from 2.7% (C3A, 1 mM) to 6.1% (C3B, 1 mM).

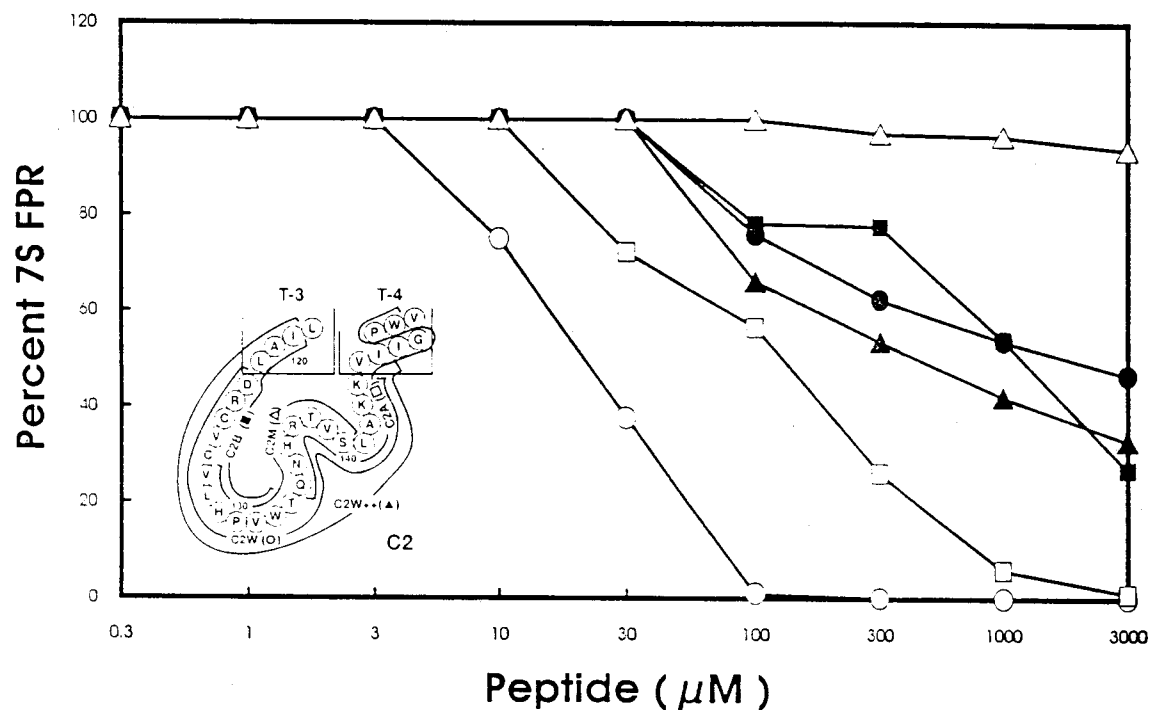


Figure 21. Comparison of the disruption of reconstituted 7S by synthetic peptides from the putative second cytoplasmic loop of FPR. Inset shows diagram of the second cytoplasmic loop and the location of various C2 peptides tested. T-3 and T-4 represent portions of the transmembrane helical regions three and four. Cytoplasmic loop C2 connects membrane domains T-3 and T-4. Numbers indicate the residue numbers in the linear sequence of FPR. The percent of total FPR sedimenting with apparent sedimentation coefficient of 7S was determined by sedimentation analysis as described in the legend for Figure 19. for each concentration indicated for several peptides whose sequence were derived or related to that of C2 loop of FPR. Peptides used are, C2W, (122-DRCVCVLHPVWTQNHRTVSLAKK-144) (○); C2W++, (119-IALDRCVCVLHPVWTQNHRTVSLAKKVI-146) (▲); revC2W, (144-KKALSVTRHNQTWVPHLVCVCRD-122) (●); C2A, (134-QNHRTVSLAKKVIIGPW-150) (□); C2B, (119-IALDRCVCVLHPVWT-133) (■); and C2M, (127-VLHPVWTQNHRTVS-140) (△). Complete inhibition would be equivalent to pure 4S form of FPR with no Gi2 added. Data points represent the mean of three different experiments and the standard error ranged from 2.3% (C2W, 30 μM) to 8.7% (C2B, 100 μM).

Earlier we reported that the middle region of the carboxyl terminal tail specifically disrupted the reconstituted 7S complex, with an IC_{50} of approximately 400 μM [15]. Additional peptides from the carboxyl terminal tail were assessed for their ability to interfere with the FPR-Gi2 complex in the present work. Figure 22 (inset) shows the COOH terminal tail peptides that were investigated. Peptide CTC, spanning the first half of CTE and containing an additional upstream sequence, was only a tenth as effective as CTE. Interestingly, peptide CTD containing the second half of CTE was not effective (data not shown). Unlike rhodopsin and the adrenergic receptors, FPR's CTB peptide corresponding to the amino terminus of the tail region had an IC_{50} of more than 3 mM, indicating only moderate activity. The CTB region in FPR does not contain a cysteine residue, which in other receptors is thought to be palmitoylated and mediate the formation of a fourth cytoplasmic loop [11]. The peptide corresponding the last 14 residues of the tail, CTZ, was inactive.

The first cytoplasmic loop of other GPCR has not previously been found to show functional involvement in G protein activation [43,53], with the recently reported exception of human muscarinic cholinergic receptor (Hm1) [54]. Interestingly, a peptide corresponding to the first cytoplasmic loop of FPR (Figure 23) was able to dissociate the reconstituted 7S with an IC_{50} of approximately 100 μM .

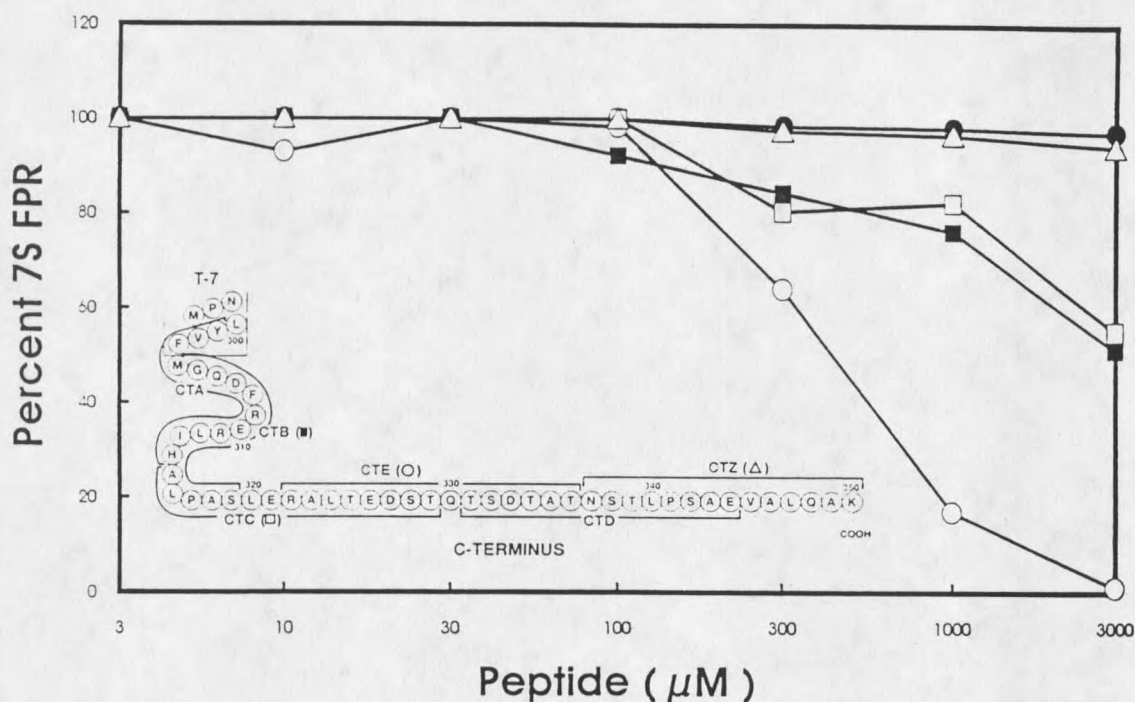


Figure 22. Comparison of the disruption of reconstituted 7S by synthetic peptides from the COOH tail of FPR. Inset shows a diagram of the COOH tail region of FPR showing the location of various peptides tested. For a description of methodology and the plot please see the legend for Figures 2 and 3. Peptides represented are CTE, (322-RALTEDSTQTSDTAT-336) (O); revCTE, (336-TATDSTQTSDETLAR-322) (●); CTC, (315-ALPASLERALTEDST-329) (□); CTB, (304-MGQDFRERLIHALPAS-319) (■); and CTZ, (337-NSTLPSTAEVALQAK-350) (Δ). Data points represent the mean of two (CTB, CTC, CTZ, revCTE) or four (CTE) different experiments and the standard error ranged from 3.6% (CTE, 100 μM) to 6.4% (CTB, 300 mM).

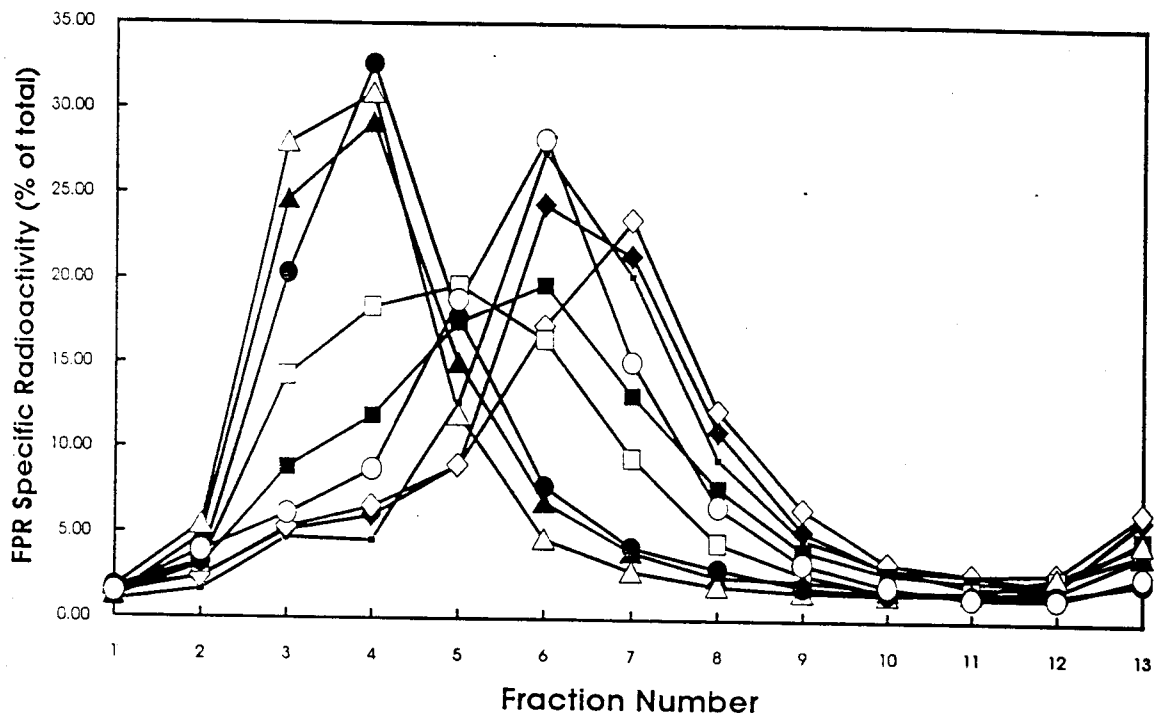


Figure 23. Effect of FPR first cytoplasmic loop peptide on the reconstituted 7S complexes. Sedimentation profile of the 4S and 7S species $\pm$ C1W peptide, (44-GNGLVIWVAGFRMTHTVTT-61), at various concentrations was plotted as a function of fraction number as described in the legends for figures 1 and 2. C1W peptide at various concentrations [1 μ M, (small $\blacksquare$); 3 μ M, ($\blacklozenge$); 10 μ M, ($\diamond$); 30 μ M, (large $\blacksquare$); 100 μ M, ($\square$); 300 μ M, ($\blacktriangle$); 1 mM, ($\triangle$)] was used to assay the disruption of the reconstituted 7S complex. Data points represent the mean of two different experiments.

Discussion

We have shown that receptor mimetic peptides, modeled after the primary structure of FPR, have specific and quantifiable effects on the ability of agonist-occupied FPR to reconstitute functional FPR-Gi2 complexes in octyl glucoside. These results suggest that the contacts between FPR and Gi2 in such preparations are extensive, involving regions in or near all three predicted cytoplasmic loops and parts of the cytoplasmic tail. Synthetic receptor and G protein peptides have been shown to interfere with functional [29,43,50,53,55] and physical [15] receptor-G protein coupling in several other systems and are believed to mimic interfacial contact sites between the two proteins. Application of site-directed mutagenesis to structurally perturb GPCR and G protein has also contributed supportive and novel information about the contact interfaces between these proteins [26]. However, our results represent a relatively comprehensive dissection of the G protein binding sites on FPR, allow us to refine current concepts about FPR structure, and to make certain predictions about the earliest events involved in the formation of the signal transduction complex.

Our current results implicate several new cytoplasmic receptor sites of interaction with Gi2 and suggest non involvement of several others cytoplasmic sites. Previously, we reported that the predicted, relatively hydrophilic mid regions of the second and third cytoplasmic loops of FPR (C3M and C2M in Figures 3 and 4) do not appear to be involved its interaction with Gi2. These results were

recently confirmed by Prossnitz and coworkers [56] using site-directed mutagenesis of these putative FPR intracellular loops. Our current results indicate, however, that stretches of the putative third cytoplasmic loop in or near the predicted cytoplasmic membrane interface are involved in Gi2 binding (Figure 20). Similar findings implicating hydrophobic amino acid receptor domains in G protein activation have been reported in the case of β_2 -adrenergic receptor. Cheung and coworkers selectively replaced the charged as well as uncharged residues in the amino terminal end of the third cytoplasmic loop and assessed the ability of mutant receptors to stimulate adenylyl cyclase and the sensitivity of receptor agonist-binding to the nonhydrolyzable GTP analogue Gpp(NH)p [45]. These authors concluded that hydrophobic interactions in the region, presumed to be an extension of the fifth transmembrane domain (analogous to C3B region of FPR), play a more critical role than ionic or hydrophilic interactions in activating Gs. In support of this conclusion, is our finding [46] that treatment of neutrophil membranes under conditions known to solubilize peripheral membrane proteins by interfering with ionic interactions did not dissociate the FPR-Gi2 complex (1 M NaCl or 5 mM EDTA). This observation suggests tight agonist-occupied FPR-Gi2 binding, presumably involving some specific hydrophobic interfaces. Taken together, these findings suggest that the more hydrophobic intramembrane extensions of the cytoplasmic domains of FPR may be accessible to G protein and important for G protein binding to the activated receptor.

Of all the peptides we have tested, the sequence corresponding to the entire second putative cytoplasmic loop (C2W, residues 122-144) is the most active with an IC_{50} of about 20 μM (Figure 21) while C2M (127-140) is inactive. In an independent study Schreiber and coworkers [57] recently found that peptide C12R (126-CVLHPVWTQNHR-137) from the second cytoplasmic loop was able to disrupt the reconstituted 7S complex with an IC_{50} of about 300 μM , which is 10 fold less effective than the C2W peptide. The inactive C2M peptide (127-VLHPVWTQNHRTVS-140) differs from the C12R peptide in that it does not contain the amino terminal cysteine (C126), but it has three additional residues at the carboxyl terminus (138-TVS-140). This difference suggests that the addition of a cysteine residue, (C126), to the amino terminus of C2M peptide may impart activity (Figure 21). Alternately, it is possible that the three additional residues on C2M peptide (138-TVS-140) may interfere with the proper folding of the peptide, rendering it inactive. These findings suggest, furthermore, that the activity conferred by the C2W peptide may reside in proximity of residues 122-126 and 141-144.

Unlike the juxtamembrane regions of the putative third cytoplasmic loop, analogous, more hydrophobic regions near the hydrophilic portions of the second cytoplasmic loop do not contribute to disrupting the FPR-Gi2 interaction. For example, the C2W++ peptide (residues 119-146, see Figure 21) containing the most hydrophilic part of the second cytoplasmic loop region plus additional hydrophobic

residues at its amino and carboxyl termini has a much weaker activity and higher IC_{50} (500 μM) than the hydrophilic C2W ($IC_{50}=20 \mu M$). In order to better localize the regions that confer activity on the putative second cytoplasmic loop, we tested two peptides corresponding to the two halves of the putative second loop which also extend into the predicted membrane regions. Peptides C2A and C2B exhibited IC_{50} 's of about 100 μM and 1 mM respectively (Figure 21). Addition of equimolar C2A and C2B simultaneously, to the reconstitution mixture did not reduce the IC_{50} for the 7S disruption below that of the more active C2A alone, indicating that the effects of these two peptides are not additive or synergistic.

These observations suggests that the second and third cytoplasmic loops probably have different types of interactions in their coupling to Gi2. The second loop may exploit electrostatic or polar interactions while the third loop may depend much more on hydrophobic domains for Gi2 binding. In support of this argument is the similarity of the C2A peptide to mastoparan (Table 7), a 14 amino acid peptide toxin from wasp venom (species *Vespula lewisii*) [58]. Mastoparan or its natural homologue mastoparan-X have been shown to mimic agonist-occupied G protein-coupled receptors to activate either Gi and Go subtypes [59-61]. Mastoparan-X has been reported to form an amphiphilic helix in phospholipid vesicles [60] or when bound to recombinant Gi1 α or Go1 α [62]. Structure-activity studies aimed at disrupting the helical secondary structure by employing amino acid substitutions at critical locations resulted in an inactive mastoparan

[59-61,63] and suggested that the formation of an amphiphilic helix is critical for mastoparan's biological activity. A high degree of sequence similarity (64%) and similarity in location of the charged residues between C2A and mastoparan analogs suggests that FPR's C2A region might interact with Gi2 by forming an amphipathic helix with charged residues on one side and the hydrophobic residues on the other.

C2A peptide	QNHRTVS-LAKKVIIGPW
Mastoparan	INLKALAALAKKIL
Mastoparan-X	INWKGIAAMAKKLL

Table 7. Comparison of the FPR C2A peptide with mastoparan and mastoparan-X. FPR residues 134-150, corresponding to the C2A peptide, was aligned with mastoparan and mastoparan-X (ref. [58]) from wasp venom. A gap in the C2A sequence was introduced to increase alignment. The single letter code for the amino acids is used. Residues that are identical or similar in at least two of the sequences are shown in bold face.

The sequence representing the first cytoplasmic loop is among the best conserved regions of G protein-coupled receptors [8,64]. Interestingly, the carboxyl terminal end of C1W peptide contains two threonines in close proximity to two basic amino acids. Threonine-56 is one amino acid from arginine-54 while threonine-58 is adjacent to histidine-57. In case of rhodopsin, a serine (S338) which is adjacent to a lysine residue has been identified as a major initial site of regulatory phosphorylation (reviewed in [65]). Two earlier functional studies, involving the first cytoplasmic loop of rhodopsin [43], and β_2 -adrenergic receptor

[53], concluded that the first loop was not involved in transducin (Gt) or Gs binding to their respective membrane receptors. In contrast, in a recent report [54] involving alanine mutagenesis scanning of human muscarinic cholinergic receptor (Hm1), a 50% reduction in agonist stimulated phosphatidylinositol release was observed when three residues from the first loop were substituted. Our results clearly indicate a concentration dependent disruption of the reconstituted 7S complex by the first cytoplasmic loop peptide with an IC_{50} of approximately 100 μ M (Figure 23).

Study of the cytoplasmic tail FPR peptides confirm our previous findings [15], and suggest further that the CTE region (Figure 22) may be the major structural determinant in the C-terminal region required in the FPR-Gi2 interaction. Peptides CTB and CTC covering parts of CTE peptide or containing upstream sequences were only 50% inhibitory at 3 mM peptide concentration (Figure 22). Schreiber and coworkers [57] found that peptides spanning the entire cytoplasmic tail as well as a fusion protein consisting the entire tail region inhibited fMLF induced ribosylation of Gi with an IC_{50} of 250 μ M peptide concentration. The fusion protein containing the entire tail competed with a $Gi\alpha$ specific antibody for binding to Gi protein with an IC_{50} of 20 μ M.

Of course, without direct structural information, it is not possible to know the precise exposure of the FPR cytoplasmic domains to the cytoplasmic surface. Thus models for the folding topography of FPR in the neutrophil plasma membrane

must still be considered within hydropathy constraints and by analogy to other receptors for which more detailed information is available. One such receptor system is that of rhodopsin in the discs of rod outer segments in retina. Dratz and Hargrave proposed [13] that the transmembrane domains of rhodopsin may form a helical bundle. This folding pattern has some experimental support [14,66]. This model suggests that accessibility of the receptor contact regions needed to "activate" guanine nucleotide exchange on G protein may require a substantial structural transition in the agonist stimulated receptor. Such a transition may involve opening of the helical bundle in the membrane in which the opening of the helices approximates the "opening of a glove" [30] accompanied by the thickening of the membrane lipids [67]. We interpret our results as supportive of this view. The fact that the most hydrophilic mid regions of the second and third cytoplasmic loops are inactive in blocking FPR-Gi2 interaction while some hydrophobic "juxtamembrane" regions are active suggests the G protein recognition of FPR does not involve the mid regions. The open glove model for the agonist-occupied receptor "catching" a G protein would at least intuitively satisfy such constraints.

The FPR transmembrane topography model proposed by us [15] originally formed the basis for our choice of potentially inhibitory peptides to be tested in the reconstitution assays. This model was based purely on hydropathy analysis and analogies with models proposed by Dratz and Hargrave [13] and by Hargrave and McDowell for other GPCR [8,9]. Two other models for FPR topography have now

been published [2,16] which differ from ours primarily in the disposition of the first and the third predicted cytoplasmic loops. Recently a model for GPCR was published by Baldwin [10] which is based on a detailed comparison of 104 G protein-coupled receptor sequences and the location of conserved and non-conserved residues in the putative transmembrane domains. Our FPR model is shown in Figure 24, panel A (termed model A) and is compared with model B after Baldwin (Figure 24, panel B). The two other FPR models [2,16] are rather similar to model B in Figure 24. Models A and B are very similar in the predicted disposition of the second cytoplasmic loop (C2) and the starting point of the C-terminal tail. The largest differences between the two models are 1) a significantly "short" C3 loop in model B (Figure 24), whose length corresponds to the inactive C3M region [15], 2) a "short" C1 loop in model B, 3) the location of two arginine residues (R201 and R205) in the transmembrane helix 5 and the location of one aspartic acid (D26) in transmembrane helix 1 of model A which is predicted to be in the polar/hydrophobic interface in model B. The presence of charged residues in the putative transmembrane regions have been previously implicated for G protein-coupled receptors [9,10,13,26] as well as various other integral membrane proteins [68-70]. Model B is perhaps more compatible with the open glove hypothesis of Dratz et.al., for the agonist-occupied receptor. Model B is also appealing because it affords a simple mechanism to transmit gross conformational changes between the cytoplasmic face of FPR and the agonist-binding pocket. G

protein binding strongly affects the affinity of the agonist fMLF for the receptor [71].

In conclusion, our work has resulted in novel observations that may have significant implications for GPCR structure and function. First, that the interaction of FPR with Gi2 must be mediated by extensive protein-protein contact involving interaction of all the cytoplasmic subdomains of FPR, including the putative first cytoplasmic loop. Second, the FPR-Gi2 interaction may well involve deep interdigitation of the two proteins, possibly including penetration of the G protein into the bilayer transmembrane helical region. Third, support for the hypothesis that occupancy of FPR by its agonist may open the "fingers" of a glove to grasp the G protein with minimal contact with the hydrophilic extremities of the FPR cytoplasmic loops. These new ramifications of FPR-Gi2 interactions provide a model to guide the design of further experiments to bring us closer to an understanding the molecular basis of chemotaxis and signal transduction.

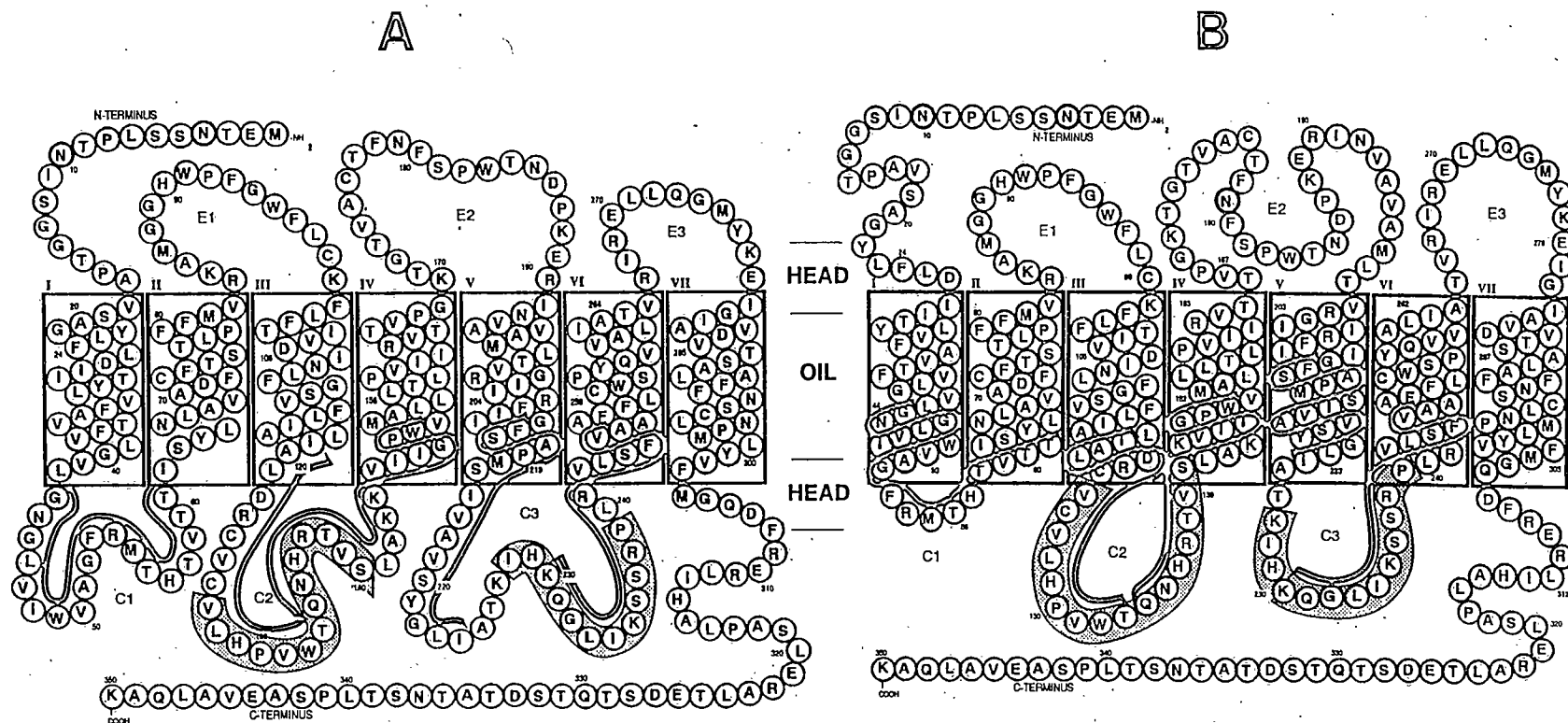


Figure 24. Models for the transmembrane topology of FPR. Model A is based on hydropathy analysis, was previously published by us (ref [15]), and is consistent with sequence alignments of other GPCR [8,9]. Model B is based on detailed alignments of conserved and non-conserved amino acids by of Baldwin [10] and is similar to other FPR models [2,16]. The regions indicated with heavy lines show some of the peptide sequences which interfere with FPR-Gi2 coupling and where the transmembrane disposition differs greatly between the two models. The regions indicated by stippled boxes highlight some of the peptide sequences that are inactive in blocking the FPR-Gi2 interaction.

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CHAPTER 6

CONCLUSIONS

Although G protein mediated pathways have generated a great deal of interest, the nature, composition and stoichiometry of the molecular species involved in transmembrane signalling have not been precisely defined. Functional interactions between receptors, G proteins and intracellular effectors are being studied using cell free systems as well as *in vivo* approaches. However, the direct physical contact between the signal transducing proteins had not been examined. Results presented in this dissertation address the fundamental hypothesis of the N-formyl peptide chemoattractant receptor (FPR) system, i.e., that FPR directly interacts with its G protein signal transduction partner to trigger neutrophil activation. In addition, the experiments described represent a novel approach to map the sites of molecular interactions of FPR with G protein which can presumably be extended to other proteins involved in neutrophil activation.

The two most significant findings presented in this dissertation are a) demonstration of a physiologically relevant, direct physical interaction between

FPR and G protein in detergent extracts free of membrane, and b) demonstration that binding of G protein to FPR involves distinct and extensive cytoplasmic domains of the receptor including regions not previously implicated in the signal transduction process.

Support for the first finding comes from the studies involving measurement of specificity, selectivity and stability of the physical association of FPR and G protein. Photoaffinity agonist-labeled, and octylglucoside-solubilized FPR retains its capability to interact both physically and functionally with G protein. The specificity of the FPR-G protein interaction is indicated by the observation that purified neutrophil G_i or bovine brain G_{i2} (>95% amino acid identity) forms a hydrodynamically distinct physical complex with FPR of apparent sedimentation coefficient of 7S. Anti- $G_{i2}\alpha$ antibodies shift the sedimentation of the 7S form deeper into the gradient indicating that the added G_{i2} protein had been integrated into 7S complex. Unrelated proteins like bovine serum albumin or irrelevant immunoglobulin do not perturb FPR sedimentation. Selectivity is evidenced by the fact that visual transducer G_t , a mixture of G_t/G_o proteins or ADP-ribosylated- G_{i2} do not reconstitute the 7S complex. As discussed in chapter 3, a range of biochemical conditions including physiological pH and salt can be successfully used to reconstitute the 7S FPR- G_{i2} complex confirming the stable nature of FPR- G_{i2} coupling.

The physiological relevance of the FPR- G_{i2} interaction in detergent solution

is supported by its response to with guanine nucleotides. GTP or its non-hydrolyzable analogue GTP γ S (but not adenine nucleotides) is able to dissociate the reconstituted 7S complex. The ability of GDP β S to dissociate the 7S complex also appears to be highly significant since it indicates that the physical coupling of FPR with Gi2 coincides with functional coupling between the two proteins. The primary function of an agonist-occupied receptor is to release the bound GDP from G protein α subunit to make the empty nucleotide site available to binding of free GTP in the cytoplasm. The fact that GDP β S can also dissociate the FPR-Gi2 complex, albeit at 80 fold less potency than GTP γ S, clearly indicates that agonist occupied-FPR in detergent empties the G α nucleotide binding site, which is the hallmark of a functional receptor-G protein complex. Since GTP is normally more abundant in the cytoplasm the empty guanine nucleotide site on the FPR-Gi2 protein complex will virtually always bind the high affinity GTP.

Because the agonist-occupied FPR can reconstitute a tight complex with G protein this presented an opportunity to map the interfacial contact regions of the two proteins. Previous investigations of receptor-G protein contact sites employed predominantly functional assays using either site-directed mutagenesis of proteins and/or blocking function with synthetic peptides. The reconstitution assay characterized and described in this dissertation was adopted to investigate the direct effects of synthetic and unmodified receptor peptides on blocking the FPR-Gi2 complex in such functional interactions. Such analysis, though unable to address

the functional consequences of FPR peptides on G protein activation, offered unique advantage by providing insights into the ensemble of contact sites required for physical molecular association between the two proteins. In addition, hydrodynamic studies of functional molecular complexes between FPR and G protein would not only supplement information obtained from activity based assays but permit us to derive new information since activity based functional assays cannot discriminate between receptor-G protein binding and activation.

From comprehensive studies employing peptides from all the predicted cytoplasmic FPR regions, the second major conclusion of this dissertation emerged. This conclusion is that the G protein binding to FPR involves extensive contact between the two proteins. Support for this conclusion comes from peptide competition studies performed on blocking reconstitution of the FPR-Gi2 complexes. Our results clearly indicate that certain FPR intracellular peptides were able to dissociate preformed 7S complexes or prevent their formation. Control peptides, however, did not perturb 7S sedimentation. For example, peptides from the FPR extracellular surfaces (E1A or E2A) and peptides that are similar in length and/or composition from unrelated proteins, for example neutrophil cytochrome b (gp91-phox) or actin were ineffective. Stereochemical selectivity was also evident in this interaction since reverse sequences of the inhibitory peptides were either inactive, or significantly diminished in their inhibitory capacity (7 to 100 fold).

This peptide analysis resulted in another major finding in this dissertation,

that the predicted three dimensional structure of FPR is either anomalous relative to hydropathy analysis or that G protein interactions with FPR extend deeply into the membrane bilayer. The evidence presented in chapters 4 and 5 indicate that the following putative cytoplasmic regions of FPR are involved in G protein binding; the first loop, the amino and carboxyl regions of the second and third loops, and the middle portion of the COOH-terminal tail. As discussed in chapter 5, results with the FPR second and third loop peptides are consistent with those obtained from functional studies involving other G protein-coupled receptor systems. The preponderance of such agreement suggests that similar structural determinants from the second and third cytoplasmic loops of other and possibly all G protein-coupled receptors may be a general feature of these systems.

Studies presented in this dissertation are on the whole consistent with the current understanding of the general architecture of receptor-G protein interactions. Certain differences, however, may reflect differences between the activity based assays and the physical reconstitution assays. Other reports utilizing the former suggest little involvement of the first loop and the COOH tail region while our results suggest the contrary. We believe that our physical reconstitution approach may discriminate between sites responsible for G protein binding to FPR as opposed to its (G protein) activation. The first unique region involved in the physical coupling of FPR-Gi2 is the first cytoplasmic loop and its juxtamembrane regions. There is supportive evidence from other systems suggesting that the first

cytoplasmic loop of human muscarinic receptor (Hm1) is involved in the **activation** of Gi proteins [1]. Since the physical interaction of other receptors with G proteins has not been adequately addressed, the role of the different cytoplasmic domains of such receptors in such interactions is not known. The involvement of the first cytoplasmic loop in G protein binding (eg. FPR) or activation (eg. Hm1 receptor) may represent a unique feature of receptors that couple to Gi type G proteins.

As is discussed in chapter 5 and in the Appendix, the serine and threonine rich region on the COOH tail (CTE region) of analogous receptors has not been implicated in G protein activation and we speculate that binding of regulatory molecules (β -arrestin like molecules or possibly actin) may affect accessibility of G protein to key activating domains on the receptors. Our laboratory has recently published evidence that the soluble 4S FPR form and its interaction with G protein may be more complex than first anticipated, possibly involving other proteins [2]. However, our evidence for physical uncoupling of FPR from FPR-actin or FPR-G protein-actin complexes is only preliminary (see Appendix). Since determination of the precise molecular composition and stoichiometry of the proteins in the 4 and 7S FPR species is beyond the scope of this dissertation, it cannot be conclusively stated that G protein can bind to such receptor-regulatory protein complexes. Moreover, any such consideration should also address the possible role of $\beta\gamma$ subunits in receptor-G protein interactions.

Without direct structural information, it is not possible to know the precise

exposure of the FPR cytoplasmic domains to the cytoplasmic surface. Thus models for the folding topography of FPR in the neutrophil plasma membrane must still be considered within hydropathy constraints and by analogy to other receptors for which more detailed information is available. One such receptor system is that of rhodopsin in the discs of rod outer segments in retina. Dratz and Hargrave proposed [3] that the transmembrane domains of rhodopsin may form a helical bundle. This folding pattern has some experimental support [4,5]. This model suggests that accessibility of the receptor contact regions to "activate" guanine nucleotide exchange on G protein may require a substantial structural transition in the agonist stimulated receptor. Such a transition may involve opening of the helical bundle in the membrane in which the opening of the helices approximates the "opening of a glove" [6] accompanied by the thickening of the membrane lipids [7]. We interpret our results as supportive of this view. The fact that the most hydrophilic mid regions of the second and third cytoplasmic loops are inactive in the reconstitution assay while the internal regions spanning some hydrophobic "juxtamembrane" regions are active suggests the G protein recognition of FPR must minimize the properties of the mid regions. The open glove model "catching" a G protein "ball" would at least intuitively satisfy such constraints.

The FPR transmembrane topography model proposed by us [8], originally formed the basis for our choice of potentially inhibitory peptides to be tested in the reconstitution assays was based purely on hydropathy analysis and analogies with

models proposed by Hargrave and McDowell for other GPCR [9,10]. Two other models for FPR topography have now been published [11,12] which differ from ours primarily in the disposition of the first and the third predicted cytoplasmic loops. Recently a model was published by Baldwin [13] which is based on comparison of 104 G protein-coupled receptors and the location of conserved and non-conserved residues in the transmembrane domains. Theoretical studies reported by Baldwin, however, while relied on prototypic receptors for visual pigments, cationic amines and neurotransmitters did not take into account several classes of structurally and functionally related receptors, for example odorant and gustatory receptors. In view of the striking diversity of the physiological events mediated by these receptor families, any sequence comparisons should be interpreted with caution.

Our FPR model is shown in Figure 24 (page 150), panel A (termed model A) and is compared with model B after Baldwin (Figure 24, panel B). The two other FPR models [11,12] are rather similar to model B in Figure 24. Models A and B are very similar in the predicted disposition of the second cytoplasmic loop (C2) and the C-terminal tail. The largest differences between the two models are 1) a significantly "short" C3 loop in model B (Figure 24), whose length corresponds to the inactive C3M region as reported previously by us [8], 2) a "short" C1 loop in model B, 3) the location of two arginine residues (R201 and R205) in the transmembrane helix 5 and the location of one aspartic acid (D26) in

transmembrane helix 1 of model A. In the later case, the position of intramembrane charged residues may seem energetically unfavorable. However, presence of charged residues in the putative transmembrane regions have been previously implicated for G protein-coupled receptors [3,10,14] as well as various other integral membrane proteins [15-17]. Model B is compatible with the glove/ball hypothesis of Dratz and affords a mechanism which will transmit a gross conformational change to the external face of FPR possibly affecting its affinity for its agonist, fMLF.

Using a functional assay involving site-directed mutagenesis in the C3M region Prossnitz and coworkers [18] confirmed our findings regarding the non-participation of the middle region of the C3 loop of FPR-Gi2 coupling. However, the authors' conclusion [18] that the "FPR C3 loop is short and that it has no role in Gi2 coupling" is **unwarranted** especially since our results clearly show that the juxtamembrane regions of C3 loop (viz. C3A and C3B) are active in Gi2 binding to FPR. In either case however, caution must be exercised in interpreting topographic models since the loop delimiting regions have not been experimentally confirmed.

In summary, this is a truly exciting time in the study of the mechanism of G protein modulated signal transduction pathways. This super family of receptors now has more than 800 members and the elucidation of the complexities of such pathways require multifaceted approaches. A great deal has been accomplished in

this dissertation towards the molecular characterization of the hydrodynamic forms of FPR. The results presented in this dissertation represent a relatively comprehensive dissection of FPR, allows us to refine current concepts about FPR structure, and make certain predictions about the earliest events involved in the formation of the signal transduction complex. Our analysis of the FPR-G protein interactions has brought us to a new level of understanding of the molecular basis of heptahelical receptor triggering of signal transduction processes.

As we gathered novel information about FPR-G protein interactions, new insights were gained into this process leading to new and compelling questions about interactions of GPCR with signalling proteins. Does the receptor utilize its transmembrane regions for recognition of proteins? How are regulatory proteins involved in the interaction? What role do the β and γ subunits of G proteins play in the interactions? Is the active form of the receptor a monomer, dimer or a multimeric form? What are the consequences of regulatory phosphorylation of proteins (involved in the signal transduction) on their physical associations?. Specifically what is the role of FPR phosphorylation in its interaction with other proteins. Does monomeric small G proteins interact with FPR? It is hoped that the convergence of techniques studying physical coupling and molecular biological technology can lead to general principles governing the structure, function and regulation of G protein-coupled receptors.

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APPENDIX: NEW PERSPECTIVES

In this Appendix, I attempt to examine the nature of the molecular composition of the detergent-solubilized FPR. FPR is normally produced from GTP γ S treated neutrophil membranes. At 4S, the apparent sedimentation coefficient characteristic of G protein free receptor is anomalous. Since FPR polypeptide chain is highly hydrophobic and requires detergent to be soluble, it is likely that the bound detergent (and endogenous lipids) decreases the average density of the FPR containing particle. Under such circumstances FPR is expected to sediment with a rate equivalent to 1-2S. Preliminary results with synthetic FPR carboxyl tail peptide CTE, and calculations of FPR molecular weight based on its hydrodynamic properties suggest that 4S FPR represents not just FPR but perhaps a heterogeneous complex of FPR with an unknown protein. As discussed below $\beta\gamma$ subunits of G proteins probably are not likely candidates but as recently reported by Jesaitis et al. [1], the unknown protein might be actin. Alternatively, the 4S form might represent a complex between FPR and β -arrestin-like or other regulatory molecule. The basis for this derives from the fact that the receptor was obtained from neutrophils stimulated by formyl peptide in the presence of cytochalasin B, a condition believed to induce regulatory phosphorylation of FPR.

[2,3]. Receptor phosphorylation and the concomitant involvement of arrestins is well documented for other G protein-coupled receptors including rhodopsin and β -adrenergic receptors (reviewed in [4,5]).

Association of $\beta\gamma$ Subunits With 4S FPR

Evidence that $\beta\gamma$ subunits may not be part of the 4S form of FPR comes from the following. Photoaffinity agonist-labeled neutrophil membranes were treated with 10 μ M GTP γ S primarily to avoid interference from endogenous G proteins in the reconstitution experiments. Such conditions are known to dissociate the heterotrimeric G proteins into α and $\beta\gamma$ subunits *in vitro*. As discussed in chapters 2 and 3, GTP γ S has no effect on the sedimentation of 4S FPR species. Moreover, anti-Gi2 α antibodies also have no effect on the 4S FPR form indicating the absence of dissociable G α subunits in the 4S species. Since the presence of $\beta\gamma$ subunits in the 4S species cannot be excluded from the above results additional reconstitution experiments were performed using purified Gi2 α and 4S FPR. Addition of large excess of Gi2 α to 4S species did not result in the formation of 7S FPR-Gi2 complexes. Since a heterotrimeric G protein is required for coupling to a receptor, the above results suggest that $\beta\gamma$ subunits are not part of the 4S species or that FPR- $\beta\gamma$ complex cannot reconstitute with Gi α . Lastly, immunosedimentation experiments need to be carried out using anti-G β or anti-G γ antibodies on the 4S species to provide clear evidence regarding the presence or

absence of $\beta\gamma$ subunits in the 4S species.

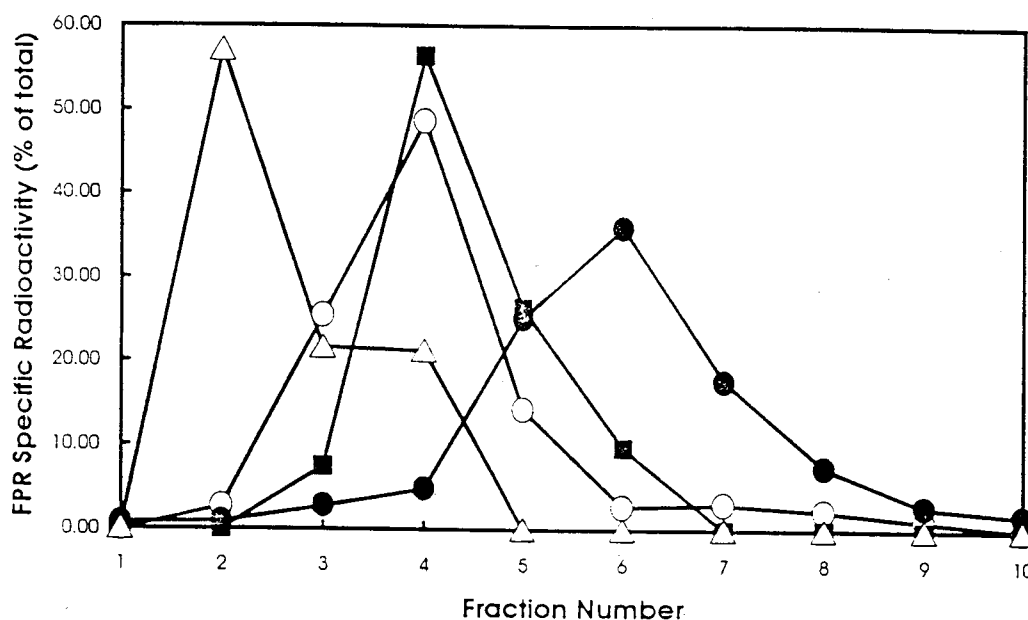


Figure 25. Dissociation of 7S FPR-Gi2 complexes to 2S FPR by CTE peptide. Reconstitution, resolution of the uncomplexed FPR and FPR complexed with Gi2 in detergent-containing linear sucrose density gradients, SDS-PAGE of FPR containing fractions and the densitometric analysis of the radioautographs was performed as described in the Materials and Methods section of Chapters 3 and 5. The receptor content of each fraction is plotted as a function of fraction number which parallel increasing sucrose concentration as described in the legend for Figure 19 (Chapter 5, page 131). The sedimentation profile of FPR alone (4S) and FPR-Gi2 (7S) are shown by (○) and (●) respectively. CTE peptide (322-RALTEDSTQTSDTAT-336) at 1 mM, (■), and 3 mM, (△) was used to assay disruption of reconstituted 7S complex. In the presence of control peptides (Table 6, Chapter 5, page 132) from the FPR extracellular face E1A and E2A, neutrophil cytochrome b peptide gp91^{phox}, Gi2 α peptide (Gi2-MKI), or from human cytosolic β -actin (actin-MKI) (not shown), the sedimentation profile was identical to that for the assay that did not contain any peptide (●). One of two experiments.

Dissociation of 4S FPR to 2S FPR by CTE Peptide

Our results with COOH tail peptides from FPR (basic experiments described in chapters 4 and 5) suggest the involvement of the middle region of the carboxyl terminal tail (CTE) region in FPR-Gi interaction. Analogous regions have not been implicated unequivocally in other systems except, for one publication involving rhodopsin-Gt interaction [6]. Recent preliminary results questions the role of the CTE region in FPR-Gi coupling. In two independent experiments, CTE peptide was able to sequentially shift the sedimentation of the 7S species to 4S (at 1 mM peptide) and then to 2S at higher peptide concentrations (>3 mM) (see Figure 25). A species identical to that of the photoaffinity labeled FPR can be clearly identified in the 2S fractions of sucrose gradients using SDS-PAGE and radioautography. Since SDS-PAGE and radioautography indicate there is no FPR degradation, we interpret these shifts as the dissociation of the 7S complex first to the 4S receptor species which then is induced to dissociate further. These results provide the first evidence supportive of the hypothesis that the 4S FPR species actually represents a complex of FPR with another yet unknown protein.

CTE peptide clearly dissociates the preformed 7S complex, and such dissociation appears to be the same as obtained with other FPR active peptides, indicating that FPR-Gi coupling may involve the CTE region. However, the CTE peptide never shows a direct effect on the 4S form of FPR up to 5 mM peptide concentration. Thus we conclude that the presence of the G protein and formation

of 7S species is required for the ultimate breakdown of the 7S FPR into the 2S FPR by CTE. In addition, none of the eighteen other FPR peptides, including the reverse sequence of CTE (up to 5 mM) or unrelated peptides disrupted the complex to the 2S form suggesting that CTE region may be the principle region involved in FPR interaction with the unknown protein. Taken together these results suggest that the added G protein somehow alters the properties of the 4S receptor form allowing CTE peptide to specifically disrupt it to a slower sedimenting 2S form.

The FPR literature supports the heterogeneity in the molecular composition of the 4S species in octyl glucoside. Allen and coworkers [7], reported that FPR obtained from unstimulated cells and in the absence of GTP has a high partial specific volume of 0.88 and 0.829 and a stokes radius of 40 and 33 angstroms when solubilized in Triton X-100 and Digitonin respectively. The authors however, did not measure the sedimentation rate of the FPR species that was used in their experiments, but reported that it eluted as a 110-130 kD species in gel filtration experiments (Figure 1A in ref. [7]). Studies in our laboratory show that GTP γ S treatment of neutrophil membranes or the detergent soluble extract is a prerequisite to obtain a G protein free-FPR form. Since the experimental conditions employed by Allen and coworkers [7] did not include guanine nucleotides, we believe that the FPR in such experiments could have been coupled to endogenous G protein. The hydrodynamic parameters (partial specific volume and the Stokes radius) mentioned above can be substituted in the following sedimentation equation [8] to

obtain calculated hydrodynamic estimate of the molecular weights of the 4S and the 7S forms of FPR.

$$M_r = \frac{s_{20,w} 6\pi\eta_{20,w} N_{av} a}{1 - V\rho_{20,w}}, \quad \text{where,}$$

N_{av} is Avagadro's number ($6.023 \times 10^{23} \text{ mole}^{-1}$)

$\eta_{20,w}$ is the viscosity of water at 20° C ($0.01 \text{ gm cm}^{-1} \text{ sec}^{-1}$)

$\rho_{20,w}$ is the density of water at 20° C (1.00 gm L^{-1})

V is the partial specific volume of FPR (0.880 ml gm^{-1} in Triton X-100)

a is the stokes radius of FPR (40 Angstroms in Triton X-100)

$s_{20,w}$ is the sedimentation coefficient of FPR (approximately 4S)

This calculation indicates that the 4S and the 7S species have apparent molecular weights of 153,000 and 267,000 Daltons. Although the amount of Triton X-100 bound to FPR is not known, the high partial specific volume (in Triton X-100) suggests that FPR is very hydrophobic and that it has a relatively large amounts of detergent and/or endogenous lipid bound to it, but it sediments at the same rate in octyl glucoside.

The hydrodynamic parameters such as partial specific volume and stokes radius for octylglucoside-solubilized G protein-free FPR are not available. Efforts

to determine them were unsuccessful (A. Jesaitis, personal communication). OG-solubilized FPR did not enter $D_2O+5\%$ sucrose, thus the partial specific volume of FPR was greater than the medium employed in the experiment. Since the partial specific volume and the stokes radius of FPR in Triton X-100 and Digitonin were similar and that the sedimentation rates of FPR in OG and Triton X-100-exchanged FPR are also similar it may be reasonable to extend molecular weight calculations performed above to OG solubilized FPR.

The possibility of excess binding of OG to FPR is unlikely because, additional OG binding to FPR should actually increase the partial specific volume of the OG-FPR complex and hence decrease the sedimentation rate. Effects of OG concentration on the sedimentation of FPR as described in chapter 3 also argues against the first two possibilities. When sedimentation runs were performed at detergent concentrations of five to ten fold below the critical micellar concentration (CMC) no significant perturbation in the sedimentation of FPR was observed (Figure 3.5). Similarly, at detergent concentrations well above the CMC no aggregation was observed. These results support our hypothesis that the 4S FPR form might represent a complex of FPR with a component that is distinct from heterotrimeric G protein.

Preliminary crosslinking studies of the 7S form of FPR (A.J.Jesaitis and D.W. Siemsen unpublished observations) revealed two independently sedimenting FPR species on SDS-PAGE, one with a molecular weight of 110 kD and the other

with 150 kD. The species that crosslinked to FPR however, have not yet been identified. Moreover, we have recently reported that the 4S complex was immunosedimentable with anti-chicken back muscle actin and anti-*Amoeba* cytoplasmic actin monoclonal antibodies suggesting that the cytoskeletal protein actin interacts with FPR [1]. Preliminary evidence (Jesaitis and Siemsen personal communication) suggests that interaction between FPR and actin could be direct. The glycosylated FPR has an apparent SDS-PAGE molecular weight of 60-65 kD while actin display a molecular weight by SDS-PAGE of about 43 kD. The molecular weight sum of these three species is 103-108 kD agreeing with the molecular weight of one of the crosslinked species (110 kD).

Interestingly, an earlier report involving expression of human FPR in *Xenopus oocytes*, required cotransfection of a complementary human factor in order to obtain a fully functional FPR [9]. The necessary complementary human factor has not been characterized further. Studies of this component might definitely shed light on the molecular composition of functional FPR species. Taken together these observations support our prediction that the 4S species in octyl glucoside probably is not FPR alone, but might represent a complex of FPR together with some unknown protein(s), possibly actin.

The Nature of the 4S Species: A New Hypothesis

The CTE region of FPR is rich in serine and threonine residues which in

analogous regions of beta-2 adrenergic receptor and rhodopsin are shown to be substrates for various kinases including receptor specific kinases [10]. There is a great deal of information to suggest that receptor kinase induced phosphorylation of such residues plays a critical role in desensitizing the response of the adrenergic and rhodopsin receptors even in the continued presence of stimulating agonist [11]. Receptor phosphorylation promotes the binding of regulatory molecules like arrestin and beta-arrestin to rhodopsin and beta-adrenergic receptors respectively [12,13], which promotes functional uncoupling of receptors from G proteins thus leading to cessation of receptor mediated signal transduction.

Two recent reports show that FPR is phosphorylated both in a time and dose (agonist) dependent manner [2,3]. Although the precise FPR residues which may act as substrates for phosphorylation are not known yet, it is possible that residues in the CTE region are also involved in the regulation of FPR mediated events. Moreover, leukocytes have been shown express to high levels of several distinct forms of β -adrenergic receptor kinases [14,15] as well as arrestin homologues [16]. Hence it is possible that the FPR mediated events might involve interactive play of kinases and arrestin homologues possibly having actin-like properties. FPR used in the experiments presented in this dissertation was obtained from fully stimulated (by agonist) neutrophils and thus might represent a covalently phosphorylated receptor. Although, the role of protein phosphatases in reversing such modifications cannot be ruled out it is certainly possible that the 4S form of FPR

is a complex with arrestin, its "actin-like" homologue or with another yet unknown regulatory molecule.

This raises the novel possibility of G protein binding to a "FPR-regulatory protein complex" (4S). Receptor kinase mediated desensitization of rhodopsin and the adrenergic receptors involves functional uncoupling of phosphorylated receptors from G proteins, but it does not exclude physical coupling of G proteins to the phosphorylated receptors. Since arrestin or its homologues are half the size of heterotrimeric G proteins, and G protein binding to receptors involves multiple contact sites (see chapter 5 for a discussion), it is possible that the regulatory protein-receptor complex can only mediate functional uncoupling of receptors and G proteins but not mediate physical uncoupling.

Thus, we hypothesize that a FPR-regulatory protein complex might still bind G protein. In such a case the interaction between G protein and the regulatory molecule might involve a region analogous to the CTE region, since CTE peptide is presumed to disrupt FPR-X-Gi interaction, where X denotes an unknown component. Sequence comparison between FPR, G protein subunits, arrestins and actin reveals a fifteen amino acid stretch on G β subunit (residues 22-36) which is 47% identical to the CTE peptide region on FPR and to certain cytoskeletal proteins such as vinculin and coronin [1].

CTE 322-RALTEDSTQTSDTAT-336
G β 022-RKACGDSTLTQITAG-036

This region on G β along with its flanking regions have been shown to be critical for a) its interaction with the G γ subunit [17,18], b) for the formation of the heterotrimeric G proteins via a triple stranded coiled coil motif [19], and c) is involved in its coupling to β -adrenergic receptor kinase (β ARK) [10].

While binding of arrestin or its homologues was shown to be specific for phosphorylated receptors [12], it has also been reported that anionic heparin or polyanions compete with the binding of arrestin to photoactivated rhodopsin [20]. These observations clearly indicate that arrestins have high affinity for polyanionic structural motifs. The CTE region upon phosphorylation (at seven potential sites) could behave like as a polyanion. Since CTE peptide used in our experiments was not phosphorylated, this may explain why a relatively high concentration (3 mM or higher) was required to obtain the 2S species. It would be useful to test a phosphorylated CTE peptide for its efficacy in dissociating the 4S and 7S complexes. If indeed the CTE peptide was dissociating FPR-"arrestin" interaction then the phosphorylated CTE peptide is expected to be a more potent agent than native CTE in breaking up 4S.

As mentioned earlier, the CTE peptide did not dissociate the 4S species up to a concentration of 5 mM, unless it were not first reconstituted with G protein to form 7S. This indicates that the interaction between FPR and the unknown protein is probably of high affinity. Moreover, since the 2S species can be obtained only from the 7S reconstituted form, this suggests that Gi somehow reduces the affinity

between FPR and the unknown protein. The proposed G_i mediated reduction in "FPR-unknown protein" affinity is then revealed by increased concentration of CTE which gains access to the interface between FPR and the unknown protein. It would be interesting to know if a phosphorylated CTE peptide can dissociate 4S species (in the absence of G_i). In addition, the apparent lack of synergy between CTE and other FPR active peptides (which independently dissociate 7S) suggests that the FPR- G_i interaction probably involves both direct (through FPR cytoplasmic loops) as well as indirect (binding mediated via a regulatory molecule).

Jesaitis and coworkers [21] proposed that during agonist mediated desensitization, FPR is segregated into microdomains which are enriched in cytoskeletal proteins (actin and fodrin) but depleted in G proteins. The authors also show [22] that translocated receptors can interact with G proteins and hence hypothesize that "lateral segregation" would represent a mechanism of controlling of receptor-G protein interactions and such reorganization of the plasma membrane, may form the molecular basis for response termination in human neutrophils.

Interactions of G protein subunits with cytoskeletal protein were documented, for example, tubulin was shown to interact with $G\alpha$ [23], as well as the $\beta\gamma$ subunits [24]. In addition, $\beta\gamma$ subunits interact and/or translocate receptor specific kinases [10], and activate various effectors including ion channels, type II adenylyl cyclase and phospholipases [25,26]. These results along with observation that there exists conserved sequence motifs between arrestins and G protein

subunits [12] provide a basis for the conjecture that arrestins may play a regulatory role between cytoskeletal proteins and FPR. Finally, the unusually high expression of arrestins and β -receptor kinases in leukocytes [14-16] as well as the demonstration that they have broad receptor specificity, regulating several G protein coupled receptors [27], further supports a potential role for the phosphorylation of FPR in its interactions with "arrestin and/or other regulatory molecules."

In summary, our preliminary results have led to the observation that the 4S from of FPR may represent a complex in itself. This observation may have significant implications for FPR mediated neutrophil activation. The nature of the 4S species should be pursued to address its composition and to ascertain its structural and/or functional roles in modulating FPR mediated events. Such molecular dissection will provide insights that will bring us closer to understand the nature of the signal transducing species involved in chemotaxis and microbicidal activity of human neutrophils.

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