



Production and purification of formyl peptide receptor : explorations of protein-protein interactions  
by Maria Renata Kohler

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in  
Microbiology

Montana State University

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**Abstract:**

Neutrophils play an essential role in fighting bacterial infections. Many neutrophil functions are the result of an interplay between cell surface receptors and their ligands. Formyl peptide receptor (FPR), through the action of its ligand, fMLF, has been shown to mediate neutrophil migratory, as well as cytotoxic functions. This receptor is believed to be organized into seven-transmembrane regions and is coupled to guanosine triphosphate-binding protein (G protein), which is a key component in FPR-mediated signal transduction. Previously, numerous attempts have been made to purify the receptor. In this work, we describe the expression and partial purification of FPR expressed in insect cells infected with recombinant baculovirus. The receptor expressed in these cells had a significantly decreased ligand binding affinity with a dissociation constant of 70 nM, as compared to the receptor on neutrophils with dissociation constant of 3 nM, suggesting that the processing of the receptor may be different. We, therefore, shifted our effort to purify FPR from Chinese hamster ovary (CHO) cells, which were found to bind ligand with a similar affinity as human neutrophils.

The purified receptor, as well as intact CHO cells expressing FPR (CHO-FPR), was used in the selection of phage peptide library in order to identify sequences that bind to the receptor. Although no consensus sequence was identified, the use of CHO-FPR cells in affinity purification of phage peptide library allowed the selection of phage that had binding characteristics different from the phage selected using wild-type CHO cells. This was demonstrated by flow cytometry, which proved to be a rapid and efficient method for screening the selected phage.

Finally, to further explore the protein-protein binding sites on FPR, and more specifically the interaction between the receptor and G protein, two cytoplasmic tail deletion mutants of FPR were constructed and analyzed for their signal transduction capabilities. Partial calcium release and suppressed chemotaxis by the deletion mutants suggest that the deleted regions are not absolutely necessary in eliciting the FPR-mediated response. Our results support the notion that there are several binding sites between FPR and G protein.

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APPROVAL

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

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Date 25 July 97

*I dedicate this work to my parents,  
Antoni and Barbara Balon, who gave  
me determination in pursuing my goals.*

Dedykuję tą pracę moim rodzicom,  
Antoniemu i Barbarze Balon,  
którzy obdarowali mnie wytrzymałością  
dobrniecia do wyznaczonych celów.  
(translated from English by Maria Kohler)

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## ABSTRACT

Neutrophils play an essential role in fighting bacterial infections. Many neutrophil functions are the result of an interplay between cell surface receptors and their ligands. Formyl peptide receptor (FPR), through the action of its ligand,  $\beta$ MLF, has been shown to mediate neutrophil migratory, as well as cytotoxic functions. This receptor is believed to be organized into seven-transmembrane regions and is coupled to guanosine triphosphate-binding protein (G protein), which is a key component in FPR-mediated signal transduction. Previously, numerous attempts have been made to purify the receptor. In this work, we describe the expression and partial purification of FPR expressed in insect cells infected with recombinant baculovirus. The receptor expressed in these cells had a significantly decreased ligand binding affinity with a dissociation constant of 70 nM, as compared to the receptor on neutrophils with dissociation constant of 3 nM, suggesting that the processing of the receptor may be different. We, therefore, shifted our effort to purify FPR from Chinese hamster ovary (CHO) cells, which were found to bind ligand with a similar affinity as human neutrophils.

The purified receptor, as well as intact CHO cells expressing FPR (CHO-FPR), was used in the selection of phage peptide library in order to identify sequences that bind to the receptor. Although no consensus sequence was identified, the use of CHO-FPR cells in affinity purification of phage peptide library allowed the selection of phage that had binding characteristics different from the phage selected using wild-type CHO cells. This was demonstrated by flow cytometry, which proved to be a rapid and efficient method for screening the selected phage.

Finally, to further explore the protein-protein binding sites on FPR, and more specifically the interaction between the receptor and G protein, two cytoplasmic tail deletion mutants of FPR were constructed and analyzed for their signal transduction capabilities. Partial calcium release and suppressed chemotaxis by the deletion mutants suggest that the deleted regions are not absolutely necessary in eliciting the FPR-mediated response. Our results support the notion that there are several binding sites between FPR and G protein.

## CHAPTER 1

### INTRODUCTION

#### **Role of Neutrophils in Immune Response**

The immune system, with its innate and acquired characteristics, plays a very important role in protecting the host from invading microorganisms. In contrast to acquired immunity resulting in mounting a response to a specific pathogen, the innate immunity involves nonspecific factors, which prevent the pathogen from entering and subsequently replicating in the host. The "noncellular" components of the innate immunity include the physical barriers formed by skin and mucous membranes, and the physiological barriers, such as pH, temperature, and various bactericidal substances. The "cellular" component of nonspecific immune response include many cells capable of endocytosis and fewer specialized cells capable of phagocytosis, such as monocytes, macrophages and neutrophils. Utilizing their exceptional migratory mechanisms, the neutrophils, which based on their morphological features are also referred to as polymorphonuclear cells (PMNs), are usually the first leukocytes to encounter the invading organism.

In the normal non-infected state, the neutrophils marginate from the center of the column of blood towards the periphery to scan for a chemotactic signal, indicating the presence of inflammation in the body. Chemoattractants are immunoregulatory substances, derived from damaged tissue cells, endothelial cells and the microorganisms themselves, which can induce immune cells to migrate along their gradients. If during margination neutrophils detect the chemoattractant, they start rolling by temporarily binding to endothelium through adhesion molecules, such as selectins. The binding of chemoattractant to its receptors on the surface of neutrophils, results in increased affinity of neutrophil integrins for the endothelial receptors. This leads to arrest of neutrophils and consequent tight adherence to endothelium followed by transmigration between the endothelial cells. The neutrophils squeeze through intercellular junctions by extending pseudopods followed by cytoplasmic streaming. Out of the circulation, the neutrophils continue to migrate through the extracellular matrix following the gradient of chemoattractants. Once the neutrophils physically encounter the bacteria, they begin the phagocytosis process followed by the formation of phagolysosome and degranulation, during which the granules fuse with the phagocytic vesicle and release their contents. The primary (azurophilic) granules contain mainly

myeloperoxidase, elastase, lysozyme and defensins, the secondary (specific) granules also contain lysozyme, in addition to collagenase and lactoferrin. The degranulation is accompanied by enhanced oxidative metabolism, which results from NADPH oxidase reduction of oxygen to superoxide anion. This radical ultimately is transformed into a variety of toxic oxygen species, such as hydrogen peroxide, hydroxyl radicals and singlet oxygen. These oxygen-dependent toxic species, along with the oxygen-independent bactericidal content of granules, render neutrophils very effective in microbial killing.

### **Formyl Peptide Receptor**

The different activities of neutrophils result from the interplay between cell surface receptors and the signaling molecules, called ligands. The receptors, acting as transducers, relay the signal across plasma membrane and mediate intracellular responses leading to specific cell activity, such as chemotaxis. The chemotactic abilities of neutrophils have been observed as early as the turn of twentieth century, however the evidence for chemoattractant receptors on neutrophils was not provided until the 1970s. Schiffmann *et al.* observed that supernatants from cultures of *Escherichia coli* had potent chemoattractant effect on rabbit intraperitoneal neutrophils (54). This

observation led to the production of short formylated peptides and the analysis of their functions as chemoattractants (58). The most potent chemoattractant formylated peptide synthesized was formyl-methionine-leucine-phenylalanine (fMLF). Interestingly, the same peptide was isolated from *E. coli* culture filtrates by Marasco *et al.* (34). Through the binding of radiolabeled fMLF, it has been shown that neutrophils have specific receptors for the formylated peptides (65). In subsequent studies, it has been determined that, in addition to chemotaxis, the formyl peptide receptor (FPR) is responsible for many other neutrophil functions associated with host protection from invading bacteria. These functions include lysosomal degranulation, superoxide production, and phagocytosis (59).

Although other chemoattractant receptors have been identified (C5aR, IL-8R, LTB<sub>4</sub>R), the FPR has been the most studied. Niedel *et al.* demonstrated that affinity-labeled receptor, partially purified from human neutrophils, electrophoresed as a broad band with an apparent molecular weight between 55,000 Da and 70,000 Da on sodium dodecyl sulfate-polyacrylamide gel, suggesting heterogenous glycosylation of the receptor (41). Niedel and coworkers subsequently further characterized the cellular and molecular characteristics of FPR in human neutrophils. They showed that the receptor can be specifically labeled with both

fluorescent and radioiodinated derivatives of formyl peptides. In addition, they were the first to successfully covalently affinity radioiodinate FPR. The deglycosylation treatments of the receptor with endo-beta-N-acetylglucosaminidase F resulted in 33 kDa polypeptide backbone, which retained its ligand binding activity, as reported by Malech *et al.* (33). They concluded that the receptor polypeptide backbone contained at least two N-linked oligosaccharide chains that were not required for ligand binding.

The studies of the effect of nucleotides on the binding of formylated peptides to FPR on neutrophil membranes led to the identification of two affinity states exhibited by the receptor (31). The addition of GTP and its nonhydrolyzable derivative, guanylylimidodiphosphate, to the membrane preparations, resulted in the conversion of high-affinity state to low-affinity state of the receptor. Through their detailed binding studies of oligopeptide chemoattractant receptor on guinea pig macrophages and macrophage membrane preparations, Snyderman and coworkers suggested that GTPase or a guanine nucleotide regulatory unit may be involved in transduction mechanisms of the receptor (30,31). Later, this nucleotide regulatory unit was referred to as GTP-binding protein (G protein).

The early knowledge of the role of G protein in signal transduction came from studies of hormone and neurotransmitter receptors and their regulation of adenylyl cyclase (for review see (4)). GTP was shown to play an essential role in receptor binding as well as in mediating effector systems. Subsequent studies led to the identification of G protein transducing systems independent of cyclic AMP generation. These systems involve, among others, photoreceptors (64) and chemoattractant receptors, including FPR (59). The G protein classification was initially based on their ability to either stimulate ( $G_s$ ) or inhibit ( $G_i$ ) adenylyl cyclase. Interestingly, it has been determined that  $G_s$  can be activated by cholera toxin (39), on the other hand,  $G_i$  can be inhibited by pertussis toxin (27). The FPR-mediated signal transduction, chemotaxis and degranulation was shown to be pertussis toxin sensitive, suggesting that the G protein that interacts with FPR is of  $G_i$  subtype (3,5,32,42).

Further characterization of FPR, as a G protein-dependent receptor, came from the identification of its DNA sequence (8). The isolation and sequencing of cDNA clones of FPR expressed in COS-7 cells revealed a translation product of 350 amino acids. The sequence was determined to have similarities with other membrane proteins containing characteristic hydrophobic polypeptide stretches proposed to form seven alpha helical transmembrane regions with an extracellular amino

terminus and intracellular carboxyl terminus. The seven transmembrane motif was first observed in bacteriorhodopsin (16). Later, this structure was shown to be characteristic of the largest family of cell surface receptors called G protein-coupled receptors (GPCR) (for review see (53)). The GPCR family include receptors for neurotransmitters, light, odorants, and more recently described chemokines. Although various GPCRs respond to different signaling agents, a model for the common chain of events of G protein-mediated signal transduction has been described (1). The ligand-occupied receptor interacts with a specific, heterotrimeric G protein, followed by the exchange of G protein-bound GDP for GTP. The GTP-bound  $\alpha$ -subunit of G protein dissociates from  $\beta\gamma$  dimer and activates a target protein leading to the production of second messengers.

The chain of events following the binding of the chemoattractants to their receptors, although not completely understood, has been examined by various groups. McPhail *et al.* described the events leading to the functional responses of phagocytic cells (36). Briefly, the binding of the ligand to the receptor initiates the activation of one or more phospholipases, which in turn mediate the production of second messengers such as 1,2-diacylglycerol, 1,2-diradylglycerol, inositol triphosphate, phosphatidic acid, arachidonic acid, and calcium ions.

These intracellular messengers mediate the activation of cellular protein kinases able to phosphorylate target proteins. Through events yet to be defined, the phosphorylated proteins regulate specific cellular responses, such as chemotaxis, degranulation or NADPH oxidase assembly. This simplified description of signal transduction is becoming more and more complex with the findings of new signaling pathways and their components. Stoyanov *et al.* demonstrated that the receptor-associated G protein may act directly on phosphoinositide-3 kinase, which leads to the generation of phosphoinositide second messengers (61). More recently, Browning *et al.* reported that fMLF stimulates the activation of nuclear factor-kappaB, which is central to the regulation of proinflammatory gene-expression (9).

Understanding FPR-mediated signal transduction events and resulting neutrophil functions is crucial for developing new strategies to prevent and to treat various clinical disorders characterized by abnormal FPR-related neutrophil functions. Neutrophils with abnormal FPR-mediated chemotactic activity have been identified in a patient with juvenile periodontitis (JP) (44), a disease of bacterial pathogenesis, which leads to alveolar bone resorption and premature tooth loss. PMNs from the JP patient failed to migrate in response to  $10^{-9}$ - $10^{-7}$  M fMLF, but exhibited normal chemotaxis upon exposure to C5a. The authors

concluded that JP neutrophils have defective FPR, however there are possibly other reasons for failed chemotaxis. One of those reasons could be a missing component of the FPR-dependent signal transduction. The role of chemoattractant receptor, has also been implicated in rheumatoid arthritis. In this autoimmune disorder, the neutrophils become activated and migrate towards chronically inflamed synovial cavities, which possibly contain formylated peptides released from injured host cells. The localization of neutrophils to the affected joints leads to the destruction of cartilage and subsequent destruction of the joint (for review see (45)).

### **Purpose of this Study**

In order to study FPR, we searched for an expression system that would allow the production and purification of functional receptor. After unsuccessful attempts to purify FPR using a baculovirus expression system (Chapter 2), we decided to use a mammalian expression system. In this work, we describe a method for the isolation and purification of FPR expressed in Chinese hamster ovary cells (CHO) (Chapter 3). The purified receptor can be utilized in various assays to study the receptor.

The undesired effects of neutrophil activation, resulting in tissue damage observed in rheumatoid arthritis, led to a search of ways, by

which this activation can be prevented. One approach is to block the binding of ligand to FPR, by designing a pharmacological agent mimicking the FPR-interacting molecule. In order to design such an agent, it is useful to determine peptide sequences, other than *f*MLF, that bind to FPR. Here, we employed phage peptide library screening using two different methods of selection of FPR-interacting peptides (Chapter 4). The first method involved the use of FPR-coupled CNBr-activated Sepharose 4B beads. The second method involved the use of intact CHO transfectants that express FPR.

Our second approach to obtain information regarding FPR-mediated signaling involved site-directed mutagenesis of the receptor. In order to examine the role of FPR carboxyl tail in signal transduction and chemotaxis, two cytoplasmic deletion mutants were generated and functionally analyzed (Chapter 5).

**CHAPTER 2****ATTEMPTS TO PRODUCE FORMYL PEPTIDE RECEPTOR  
USING BACULOVIRUS EXPRESSION SYSTEM****Introduction**

The baculovirus expression system has been widely used for large scale production of various proteins including membrane receptors. Narayan and coworkers (40) were able to express functional rat lutropin/choriogonadotropin receptor (LH/CG-R) in Sf9 insect cells using the baculovirus expression system. LH/CG-R is a member of the G protein-coupled receptor family. The functionality of the receptor was determined by the detection of increased intracellular concentration of cAMP in response to high affinity binding of human choriogonadotropin.

Baculovirus, also known as *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV), produces two viral forms during infection: extracellular virus particles (ECV) and occlusions made mainly from the polyhedrin protein. These occlusions, also called polyhedra, protect and transmit the virus. A viral gene encoding the polyhedrin protein is nonessential for infection or replication. This gene can be replaced with a gene of interest and will result in an occlusion negative virus. There

are several expression vectors that are designed for effective protein expression and purification using the baculovirus expression system.

In this study, we used an expression vector pBlueBacHis coding for six histidine residues upstream from the initiator methionine of FPR (Fig.1). Histidine and tryptophan residues are known for their metal ions chelating properties which can be utilized in protein isolation. The histidine tagged proteins can be separated from the rest of the solubilized membrane proteins on a metal affinity column. The immobilized material can then be eluted from the column by either lowering the pH or by treatment with imidazole. The pBlueBacHis vector also contains an enterokinase cleavage site downstream from the polyhistidine sequence. This cleavage site can be used during purification to remove the histidine tag.

In order to use the purified protein for further experiments, it is necessary to determine if the expressed protein is properly folded. In case of cell surface receptors, a proper transport of the molecule to the plasmalemma and a normal ligand binding affinity are good indicators of proper protein folding. The fluorescence activated cell scanner (FACScan) can be utilized to calculate the dissociation constants of fluoresceinated formylated peptides to FPR expressed on the surface of Sf9 cells.

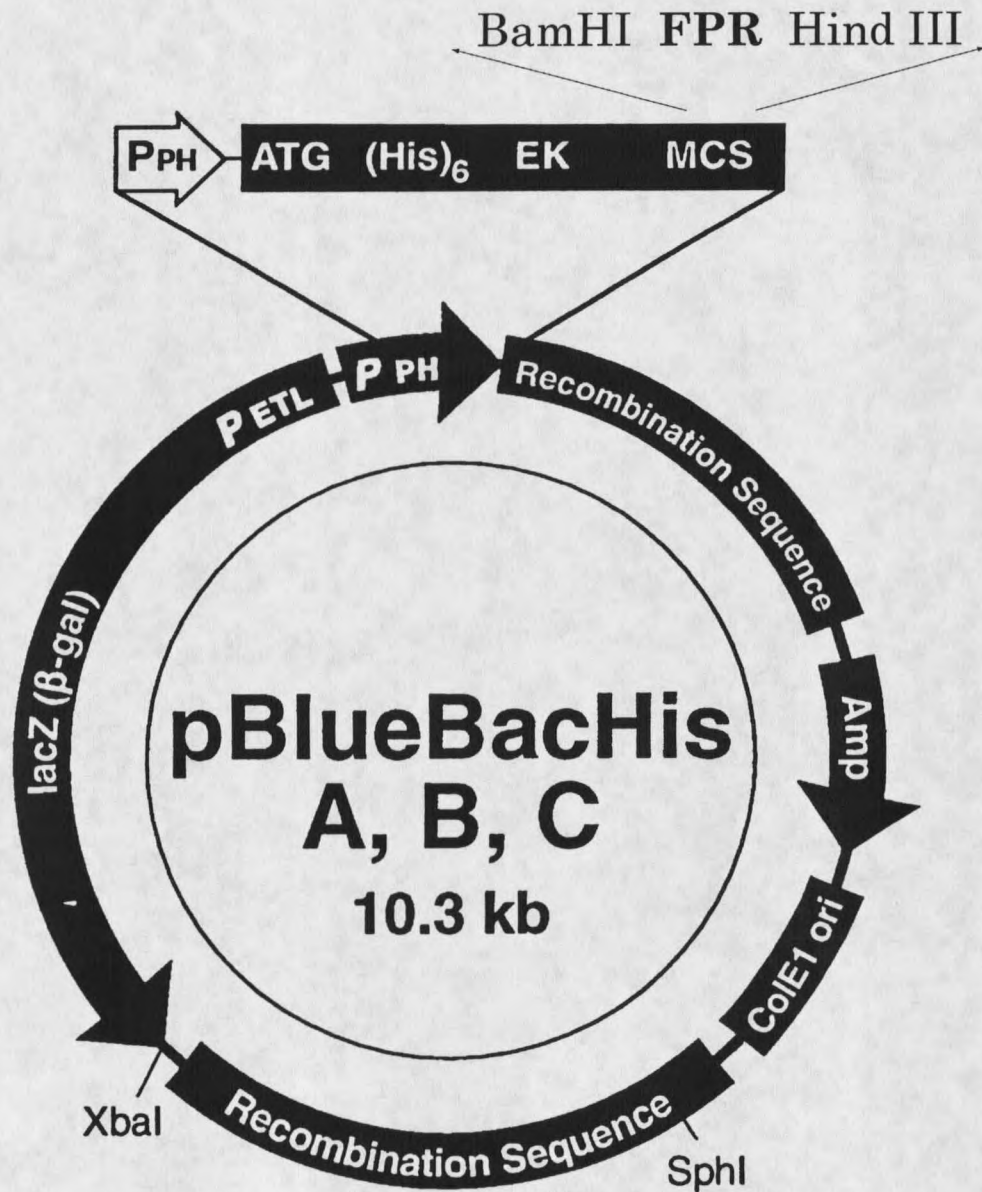


Fig. 1. Schematic figure of the pBlueBacHis vector. The FPR cDNA was inserted into the BamHI-HindIII cloning site of reading frame B. Adaptation from a figure supplied by Invitrogen (San Diego, CA).

## Materials and Methods

### Cell Lines and Antibodies

Sf9 cells are derived from the pupal ovarian tissue of the fall army worm, *Spodoptera frugiperda*. These cells double about every 24 hours and can either be grown in monolayers or in suspension in complete TNM-FH insect medium (Sigma, St. Louis, MO) supplemented with heat inactivated fetal bovine serum. They grow best at temperatures between 27 and 30°C. Anti-FPR serum was produced by immunizing a rabbit with a peptide encoding the carboxyl-terminal 14 amino acids of FPR coupled to Keyhole limpet hemocyanin. The anti-FPR antiserum was further purified using the carboxy-terminal peptide coupled to cyanogen bromide-activated Sepharose beads.

### Production of Recombinant Baculovirus

Polymerase chain reaction (PCR) was performed to amplify the FPR cDNA (kindly provided by Dr. Richard Ye, Scripps Research Institute, La Jolla, CA; 47). The primers were constructed with overhanging *Bam*HI and *Hind*III restriction sites. The FPR cDNA sequence was then inserted into the transfer vector, pBlueBacHisB (Invitrogen, San Diego, CA) linearized at *Bam*HI and *Hind*III sites. This vector allowed the FPR insert

to be ligated in frame. The pBlueBacHisB with the insert was then transfected into Sf9 cells along with commercially acquired linear AcMNPV DNA using the cationic liposome technique (18). Through homologous recombination, the FPR sequence was inserted into the viral DNA.

### **Recombinant Plaque Purification**

To obtain plaques, the Sf9 cells were plated to cover about 50% of the plate surface and the recombinant baculovirus was added at multiplicity of infection (MOI) 5. After a sufficient amount of time allowed for infection (approximately 1 h), the cells were overlaid with agarose. In 5-7 days the plaques started to appear. The addition of chromogenic dye 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal) to the plates produced a blue color around the plaques indicating successful infection by the recombinant virus which allowed the production of  $\beta$ -galactosidase by the cells. The selected plaque lifts were used to infect fresh cells and the expression of FPR was determined by immunofluorescence. Recombinant baculovirus that resulted in the highest expression level of FPR was propagated for further experiments.

### **Immunofluorescence Staining of FPR Expressed in Sf9 Insect Cells**

The infected cells were plated on coverslips treated with poly-L-lysine. They were allowed to attach for approximately 30 min and fixed for 2 h in 2.5% paraformaldehyde in phosphate buffered saline solution (PBS). Cells were permeabilized with 0.01% saponin and non-specific binding was blocked by 0.2% gelatin. Prepared in this way cells were incubated first with anti-FPR antibody and then with fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit antibody. Cell-bound fluorescence was observed with a fluorescence microscope.

### **Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis**

The Sf9 cells were infected with the recombinant baculovirus and 1 ml aliquots were removed at different time points. The cells were pelleted and resuspended in 20 mM PBS. After 4 cycles of freeze-thaw treatment, the insoluble proteins were pelleted by microcentrifugation (14,000 rpm, 10 min). Both, the pellets and the supernatants were resuspended in Laemmli buffer and loaded onto a 10% polyacrylamide gel. After the completion of the electrophoresis, the proteins were transferred onto a nitrocellulose filter. The filter was blocked with Blotto Tween (5% non-fat

milk, 0.2% Tween 20 in PBS pH 7.4), incubated in diluting buffer (3% normal goat serum, 1% BSA, 0.2% Tween 20 in PBS pH 7.4) with anti-FPR polyclonal antibody (1:200) overnight at 4°C, washed with wash buffer (250 mM NaCl, 10 mM HEPES pH 7.4, 0.2% Tween 20 in dH<sub>2</sub>O), incubated with an alkaline phosphatase conjugated secondary antibody (1:2000), 1 h at room temperature, washed and developed using the 5-Bromo-4-chloro-3-indolyl phosphate/Nitroblue tetrazolium (BCIP/NBT) kit according to manufacturer's recommendations (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

#### **Preparation of Sf9 Membranes and Extraction of FPR.**

Sf9 cells were infected for 48 hours and rinsed in Hanks' Balanced Salts solution (Sigma, St. Louis, MO). They were then resuspended in Relax (+) (10 mM HEPES pH 7.4, 100 mM KCl, 10 mM NaCl, 3.5 mM MgCl<sub>2</sub> and 1 mM ATP) and a pressure of 400 psi was applied for 15 minutes to lyse the cells. The cavitate was centrifuged at slow speed (900 x g, 15 min) to remove cell nuclei and some organelles. The remaining supernatant was centrifuged at (228,000 x g, 30 min), to pellet cell membranes. A similar method for preparation of neutrophils and fibroblast membranes was described previously by Schreiber and coworkers (56). The membranes were resuspended in phosphate buffer

(20 mM, pH 7.8) and 1 mM phenylmethylsulfonyl fluoride (PMSF) with addition of either 60 mM octylglucoside (OG) or 1% Triton X-100 (TX-100), in the presence or in the absence of 1M NaCl. After ultracentrifugation (900 x g, 5 minutes), the obtained pellet (insoluble proteins) and supernatant (containing extracted FPR) were subjected to SDS-PAGE and Western blot analysis.

**FACScan Analysis of Formyl  
Peptide Binding to FPR  
Expressed in Sf9 Cells**

The cells were infected (MOI 5) for 48 hours. After washing, the cells were resuspended in PBS containing  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , and 5% FBS followed by incubation (40 min on ice with occasional mixing) with different dilutions of fNleLFNleYK-fluorescein derivative (fluorescein-labeled analog of fMLF) and FACScan analysis. To measure unspecific binding, parallel tubes were incubated with a 1000-fold excess of fMLF. Specific binding was calculated as total binding subtracted by binding in the presence of fMLF. The dissociation constant ( $K_d$ ) was determined by least squares analysis of the mean fluorescence using the GraphPad Prism software.

## Results

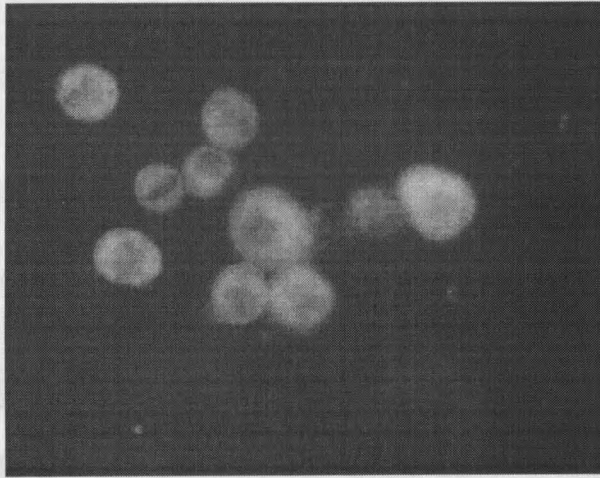
### Immunofluorescence of FPR

Fixed and permeabilized Sf9 cells were treated with anti-FPR antibody followed by FITC conjugated secondary antibody. There was a visible difference between the uninfected Sf9 cells and the cells expressing FPR (Fig.2). The labeled FPR seemed to be evenly distributed throughout the cells, which made it difficult to determine if the expressed receptor was properly inserted into the membranes of Sf9 cell.

### Time Course for Maximal Expression of FPR in Sf9 Cells

Infected Sf9 cells were lysed and the resulting solubilized (supernatant) and not solubilized (pellet) proteins were subjected to gel electrophoresis. Western blot was performed in order to determine a time point at which the Sf9 cells produce the most FPR. In addition to the band migrating around the expected 69 kDa, there was also a band appearing just above the 28 kDa molecular weight standard (Fig.3). A band with a similar relative mobility was reported earlier by Quehenberger and his coworkers (49) as an unglycosylated form of FPR. This band was not present in the negative control (uninfected cells) (not

A



B

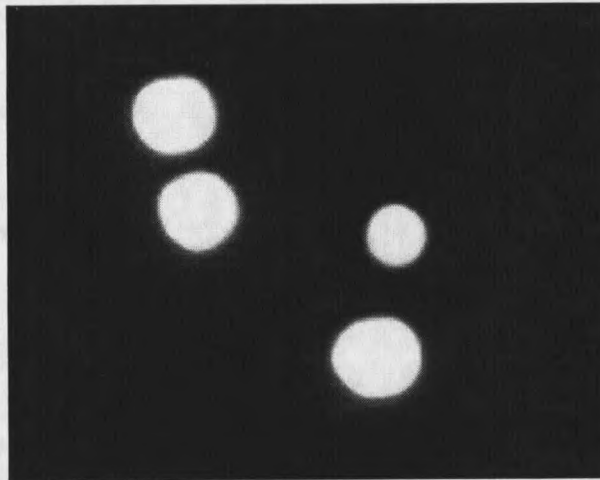


Fig. 2. Immunofluorescence analysis of FPR expressed in Sf9 cells. A. Uninfected Sf9 cells (control). B. Cells infected with recombinant baculovirus. Uninfected and infected cells were treated identically; the cells were fixed and permeabilized, incubated with an antibody against FPR and a secondary fluorescein-conjugated antibody. The difference in the backgrounds of the two photographs is due to the exposure time (20 s for A and 2 s for B) being compensated for by the camera, i.e., if the background in A would be as dark as in B, the staining of the uninfected cells would be barely visible.

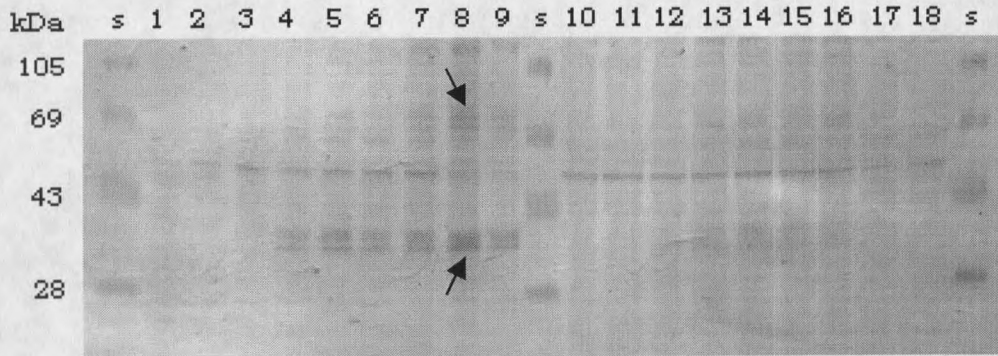


Fig. 3. SDS-PAGE and Western blot of Sf9 cells infected with recombinant baculovirus (His-FPR). The cells were infected for 18 h (lanes 1 and 10), 24 h (lanes 2 and 11), 30 h (lanes 3 and 12), 42 h (lanes 4 and 13), 45 h (lanes 5 and 14), 49 h (lanes 6 and 15), 53 h (lanes 7 and 16), 67 h (lanes 8 and 17) and 73 h (lanes 9 and 18) and lysed by freeze-thaw treatment. The lysates were resuspended in 20 mM PBS and sedimented. Lanes 1-9 represent pellets after extraction and lanes 10-18 represent extraction supernatants. The top arrow points towards the putative glycosylated form of FPR. The bottom arrow points towards the putative unglycosylated form of FPR. Based on the blot, the two forms of FPR are best expressed at 67 hours post infection (lane 8). s = molecular weight standard.

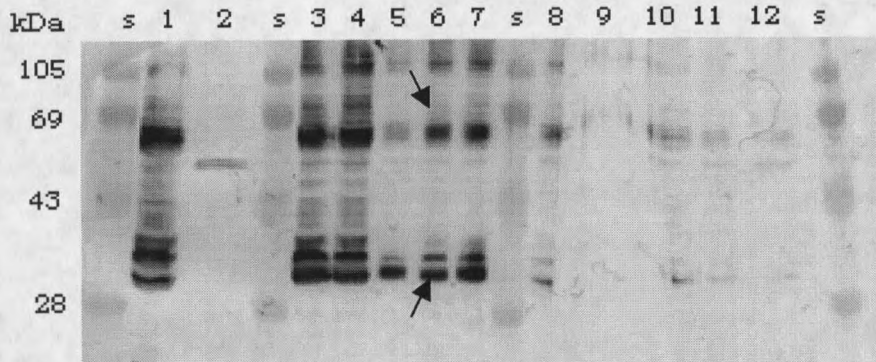


Fig. 4. SDS-PAGE and Western blot of membrane preparation (mp) of Sf9 cells infected with recombinant baculovirus, after extraction under various conditions: lane 1 - mp low speed pellet, lane 2 - mp high speed supernatant, lanes 3 and 8 - buffer suspension of cell membranes (high speed pellet), lanes 4 and 9 - extraction with 1% TX-100, lanes 5 and 10 - extraction with 1% TX-100 and 1M NaCl, lanes 6 and 11 - extraction with 30 mM OG and lanes 7 and 12 - extraction with 30 mM OG and 1 M NaCl. Lanes 3-7 represent pellets after extraction, lanes 8-12 represent extraction supernatants. The top arrow indicates the putative glycosylated form of FPR. The bottom arrow indicates the putative unglycosylated form of FPR. Based on the blot, the FPR appeared mostly in the final pellet (lanes 3-7) indicating unsuccessful extraction from the membranes of Sf9 cells. s = molecular weight standard.

shown). The maximal protein expression was established to occur between 45-67 hours p.i.

### **Extraction of FPR from the Membranes of Sf9 Cells**

It is necessary to be able to extract the FPR from the membranes of the Sf9 cells before it can be purified and used in further experiments. The following experiment was carried out to examine the solubility properties of FPR expressed in insect cells. The Sf9 cells were infected for 48 h and the membranes were prepared by N<sub>2</sub> cavitation. Various FPR extraction conditions were carried out in order to determine the most effective one. Western blot of the extract showed a band slightly below the 69 kDa marker (Fig. 4), which corresponds to the glycosylated form of the receptor. However, it appeared mostly in the final pellet, which indicated that the receptor was not successfully extracted from the membranes of the insect cells.

### **FACScan Results**

The FACScan results indicated that the binding affinity of FPR expressed in Sf9 cells with  $K_d$  of 70 nM was significantly lower than the binding affinity of FPR expressed in neutrophils ( $K_d=2.7-40$  nM) (48). Several factors could have contributed to the low binding affinity. The

difference in posttranslational modification in insect cells as compared to neutrophils, as well as improper folding of FPR in those cells, could have led to altered glycosylation and lower binding affinity. Finally, Fay *et al.* (17) have shown that the uncoupling of the receptor from G protein results in two orders of magnitude decrease in ligand binding affinity as compared to the binding affinity of the receptor associated with G protein. It has been also reported (50) that the G<sub>i</sub> proteins are absent in the Sf9 cells. This could possibly lead to a lower ligand binding affinity of the FPR expressed in Sf9 cells, which we and others (50) have observed.

### **Discussion**

The baculovirus expression system has been effectively used for the production of large amounts of mammalian proteins. Many researchers report successful expression and subsequent extraction of various membrane proteins such as the rat olfactory seven-transmembrane-domain receptor (21) and the human epidermal growth factor receptor (23). Although, we have observed the expression of FPR in Sf9 cells, we were not able to effectively extract the receptor from those cells. It has been previously shown by Klotz and Jesaitis (29) that the energy depletion of human neutrophils decreased the association of FPR with the structures underlying the plasma membrane possibly making

the FPR extraction easier. In their work the depletion of ATP was achieved by the pretreatment of neutrophils with NaF prior to membrane preparation. Perhaps pretreating the Sf9 cells with NaF would allow more effective extraction of FPR. Another method, shown by Iizuka and Fukuda to be effective in the extraction of the bovine nicotinic acetylcholine receptor  $\alpha$ -subunit from baculovirus infected Sf9 cells, was the extraction with Zwittergent (25). This method could be investigated with FPR extraction.

The FACScan analysis revealed that the FPR expressed in Sf9 cells did not bind ligand efficiently. It has been shown that large complex-type oligosaccharides, which are produced by vertebrate cells, are absent in insects cells (24). This could have lead to the altered glycosylation of the receptor and consequent lower ligand binding affinity. A possible misfolding of the FPR expressed in Sf9 cells could have also contributed to the altered dissociation constant of the receptor. Furthermore, the absence of  $G_i$  proteins in the Sf9 cells, as reported by Quehenberger *et al.* (50), can lead to a single low binding affinity of the receptor as opposed to G protein-dependent low and high binding affinities of fMLF observed in neutrophils (30).

Due to problems in purification of His-FPR from Sf9 cells as well as altered ligand binding we decided to investigate the possibility of FPR production in a mammalian expression system.

### CHAPTER 3

## PURIFICATION OF FORMYL PEPTIDE RECEPTOR EXPRESSED IN CHINESE HAMSTER OVARY CELLS

### Introduction

Early developments in techniques of mammalian tissue culture and subsequent cell culture date back to the beginning of the 20th century. Sanford *et al.* (52) investigated the possibility of obtaining a pure, C3H mouse L cell culture starting with just a single cell. They were able to isolate single cells from a mixed population of cells, that originated from connective tissue explant, and support their growth and proliferation. Since then, mammalian cells have been used in many research applications such as vaccine production, viral propagation, hybridoma generation, as well as the production of desirable transformants.

It has been previously reported that FPR can be functionally expressed in transfected mammalian cell lines (47). In our further experiments, we have used Chinese hamster ovary (CHO) cells, which have been used extensively for the expression of recombinant proteins. The CHO cell line expressing FPR was generated in our laboratory by Dr.

Miettinen. The ligand binding of FPR expressed by CHO cells was determined by flow cytometry. The CHO-FPR transfectants exhibited a dissociation constant ( $K_d$ ) of approximately 4 nM for binding of  $\beta$ NleLFNleYK-fluorescein (Miettinen, unpublished), which is comparable to the high affinity  $K_d$  of neutrophil FPR of 1-3 nM, as reported by Prossnitz *et al.* (48).

## Materials and Methods

### Cell Lines and Antibodies

The human FPR cDNA sequence was ligated into pBGSA plasmid (62) and transformed into *E. coli* Top 10. A plasmid containing the insert in the proper orientation, as verified by restriction mapping, was transfected into CHO-wild type (WT) cells. Geneticin (G418) resistance, conferred by the plasmid, was employed in the selection of cell clones. CHO transfectants expressing FPR were grown in monolayers in  $\alpha$ -modified Eagle's medium (Sigma, St. Louis, MO) supplemented with 5% FBS, 50 U/ml penicillin and 50  $\mu$ g/ml streptomycin. They were maintained with 0.5 mg/ml Geneticin (G418). To induce increased expression of FPR, 6 mM sodium butyrate was added to the medium 16-24 hours before each experiment (22). Anti-FITC antiserum was

produced by injecting rabbits with fluorescein linked to keyhole limpet hemocyanin. The antiserum was further purified on anti-FITC column.

### **Preparation of CHO-FPR Membranes and Extraction of FPR**

Near confluent cells grown on 15 cm tissue culture plates were placed on ice and rinsed 3 times with cold PBS before the addition of 100 nM  $\beta$ MBenzoylphenylalanineFYK-fluorescein peptide ( $\beta$ MBpaFYK-fluorescein, a generous gift from Dr. Mills). This photoaffinity analog of  $\beta$ MLF was used to label FPR and to permit the detection and the purification of the receptor. The cells were incubated for 10 min on ice and subjected to UV light for 15 min to allow for photocrosslinking, during which a covalent bond was formed between the receptor and the peptide. After the crosslinking, the cells were incubated for 10 min with addition of 10  $\mu$ M  $\beta$ MLF, to compete off the non-specifically bound ligand and ligand that was not crosslinked, 1  $\mu$ g/ml leupeptine and 200  $\mu$ M DTT. Next, the cells were removed from the plates by scraping and sedimented by centrifugation (100,000 x g, 30 min, 4°C). The pellets were resuspended in 1 ml of high salt buffer (0.8 M NaCl, 50 mM Na<sub>2</sub>CO<sub>3</sub> pH 10-11, 200  $\mu$ M DTT, 1  $\mu$ g/ml leupeptine), sonicated and subjected to another highspeed spin (100,000 x g, 30 min, 4°C). This procedure was repeated and the new pellets were resuspended in PBS containing 200

$\mu$ M DTT and 1  $\mu$ g/ml leupeptine, sonicated and centrifuged (100,000 x g, 30 min, 4°C). The resulting pellet was stored at -20°C for later use.

**Octyl Glucoside Extraction of FPR and Purification by High Performance Liquid Chromatography (HPLC)**

The membrane pellets were resuspended in 5 mM HEPES, pH 7.4, 3% OG, 200  $\mu$ M DTT and 1  $\mu$ g/ml leupeptine, sonicated and sedimented (100,000 x g, 30 min, 4°C). The supernatant containing solubilized membrane extract was subjected to HPLC by running through anion exchange column VYDAC-300UHP575P followed by gel filtration column TSK G3000SW. The buffers used were: Buffer A; 5 mM HEPES, pH 7.4, 1% OG, 0.02 % NaN<sub>2</sub> and Buffer B; 2 M ammonium acetate. The run was carried out at a 97:3 ratio of Buffer A and Buffer B. Total protein was monitored by observing emission intensity at 340 nm with excitation at 280 nm. The fluorescence was monitored by excitation at 490 nm and emission at 520 nm. The samples were collected in 30 second fractions (beginning with the samples showing increased fluorescence) and analyzed on a fluorimeter to determine the ratio of tryptophan (by excitation at 280 nm and emission at 340 nm) to fluorescein (by excitation at 490 nm and emission at 520 nm) indicating the purity of extracted FPR.

### **Western Blot and Silver Stain Analyses of HPLC Fractions**

The HPLC fractions were concentrated using centricon 30 (Amicon, Beverly, MA) and 4x Laemmli sample buffer was added. Two identical sets of samples were run on a 10% SDS-polyacrylamide gel. One set was transferred to nitrocellulose and Western analysis was performed as described before (Chapter 2) except for the primary antisera, which in this experiment was anti-fluorescein antiserum (1:200). The second set of samples was used for the silver stain analysis. The gel was fixed for 1 h in 50% MeOH and 12 % acetic acid, washed 3 times (20 min) with 50% EtOH, soaked in thiosulfate solution (0.2 g/l) for 1 min, washed 3 times (30 s) with dH<sub>2</sub>O, soaked in silver solution (2 g/l silver nitrate, 1 ml/l formaldehyde) and washed 2 times (20 s) with dH<sub>2</sub>O. Next, the gel was developed until bands became visible (2-3 min) and the reaction was stopped with Coomassie destain (25% isopropanol, 10% acetic acid).

## Results

### **HPLC Purification of fMBpaFYK-fluorescein Photocrosslinked FPR from Transfected CHO Cells**

In order to purify FPR, the CHO-FPR cells were incubated with a fluorescein-conjugated photoaffinity analogue of fMLF. The crosslinking of the ligand to the receptor was induced with UV light and the photocrosslinked FPR was extracted from the isolated membranes with OG. In order to isolate the FPR from cell extract, an anion exchange followed by a gel filtration, in an HPLC system, were utilized. Most solubilized membrane proteins were retained on the anion exchange column, allowing the positively charged proteins, including FPR, to flow through. In the tandem gel filtration column the eluted proteins were then separated by size. According to the differential distributions of fluorescein and tryptophan (Fig. 5A), the fractions containing FPR were eluted from the column just before the major protein peak (Fig. 5B). The second HPLC run, carried out with fluorescein peak fractions of the first tandem anion exchange, gel filtration run, revealed only one peak of total protein which corresponded to the fluorescence peak (Fig. 6). To obtain control (non-FPR) fractions, used for later experiments, the WT cell membranes were also subjected to two HPLC runs and fractions

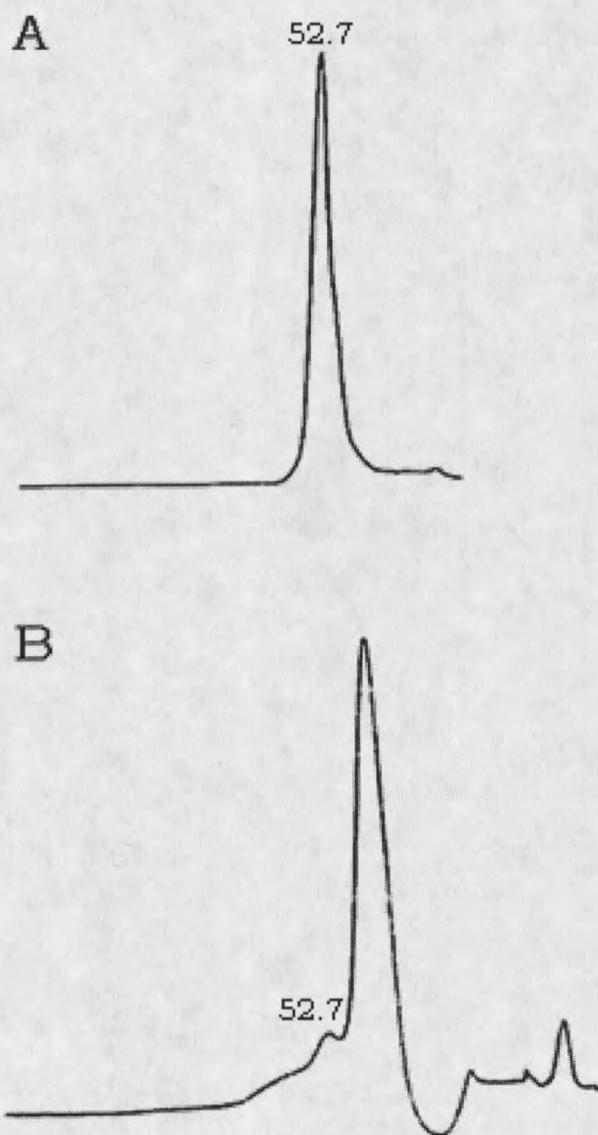


Fig. 5. HPLC of photocrosslinked FPR extracted from CHO cells. OG membrane extract was purified on anion exchange column followed by gel filtration column. A. Fluorescein fluorescence in the elution profile identifies fluorescein-labeled molecules, presumably mostly FPR. B. Tryptophan fluorescence in the elution profile identifies total protein. The numbers indicate the times (min) of elutions of the marked peaks.

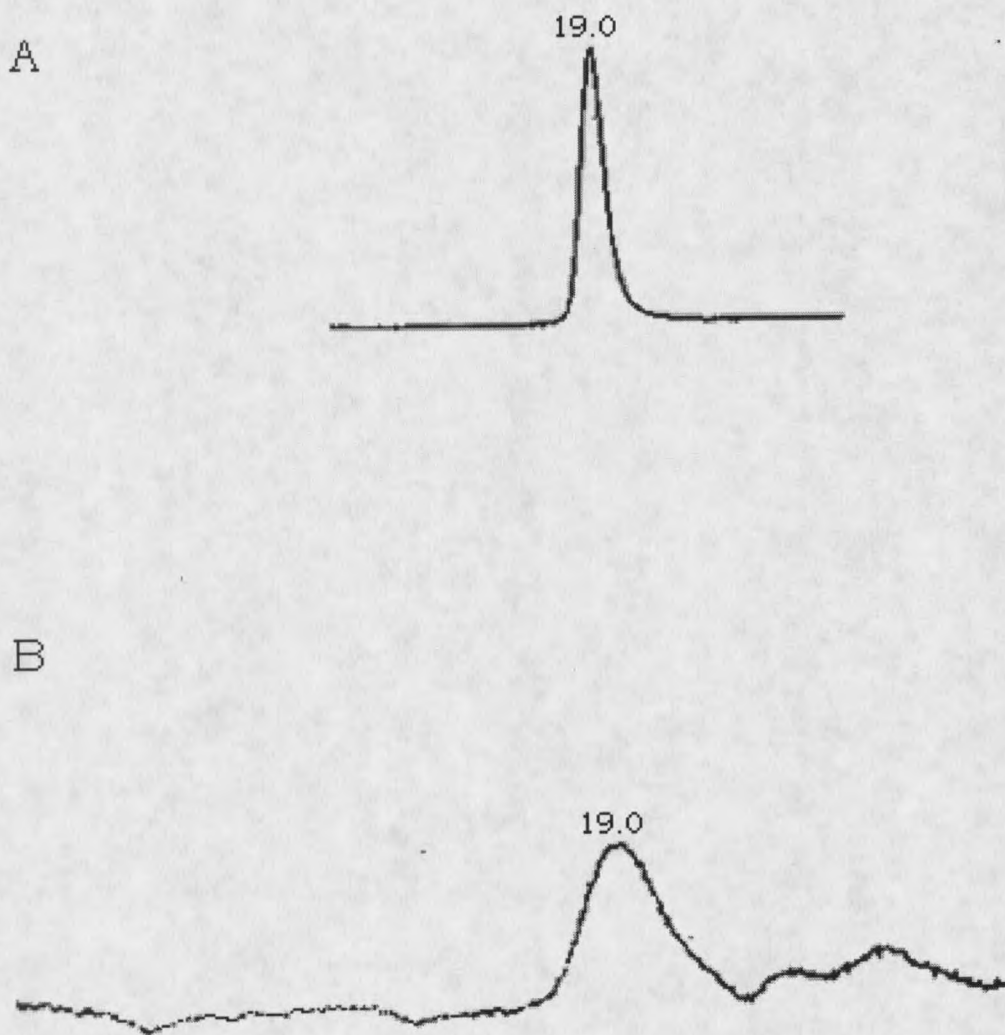


Fig. 6. Second step in HPLC purification of FPR. The fractions eluted from the first HPLC run corresponding to the fluorescein fluorescence peak were subjected to a second HPLC run. A. Fluorescein fluorescence. B. Tryptophan fluorescence. Based on the differential distribution of fluorescein and tryptophan, total protein fractions contain mostly fluorescein-labeled FPR. The numbers indicate elution times (min) of the marked peaks.

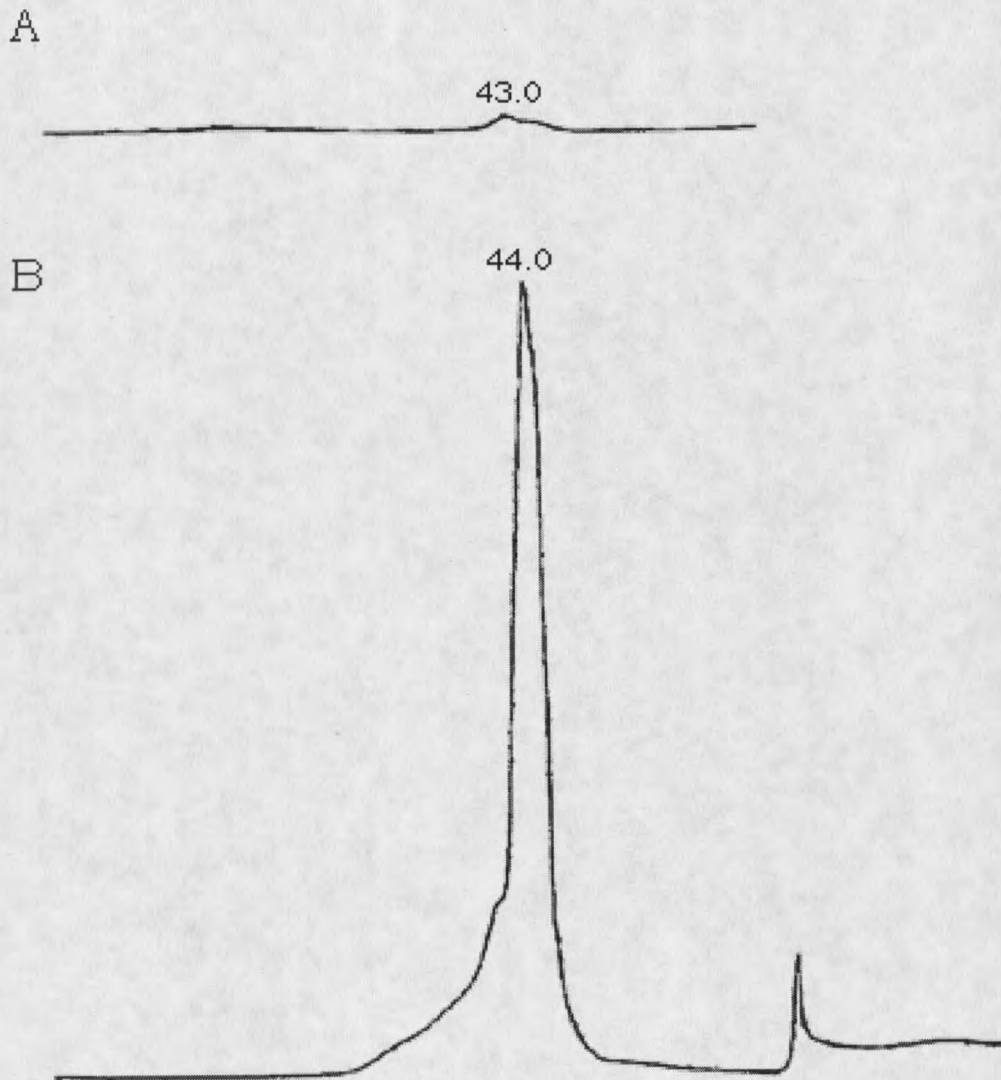
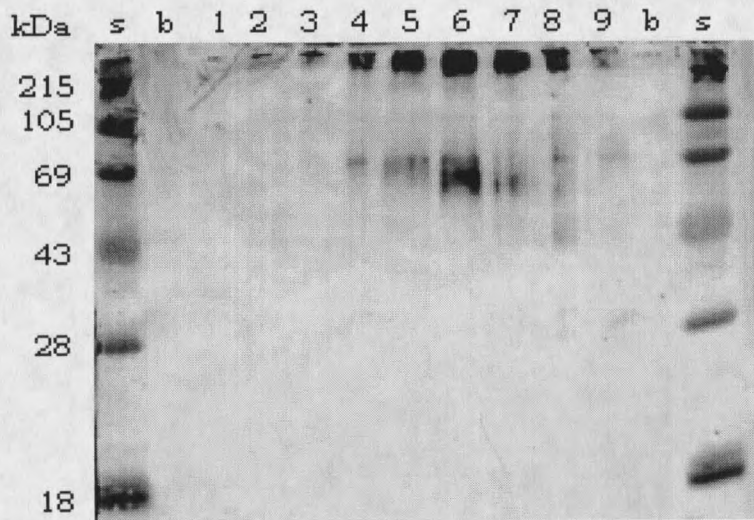


Fig. 7. HPLC of membrane extracts of CHO-WT cells. A. Fluorescein fluorescence. B. Tryptophan fluorescence. The differential distribution of fluorescein and tryptophan indicate that the fluorescein fluorescence is associated with the total protein fraction. The numbers indicate the times (min) of elutions of the marked peaks.

A



B

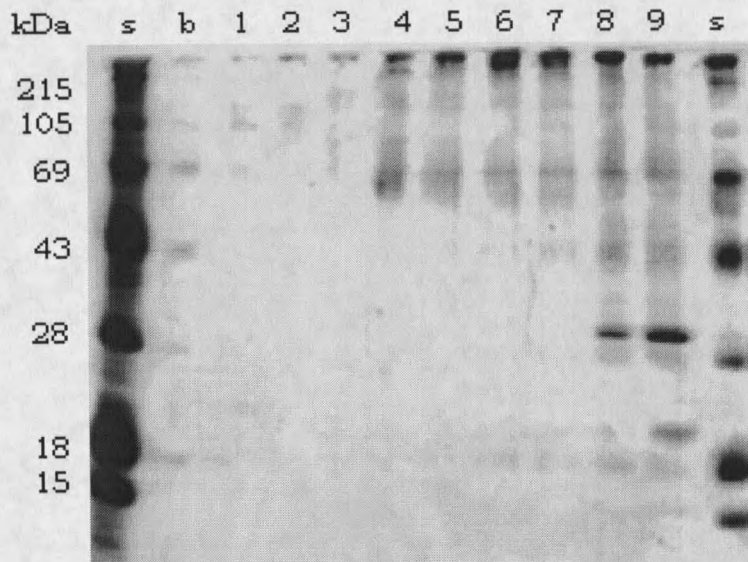


Fig. 8. SDS-PAGE of FPR extracted from CHO cells and purified by HPLC, as described in the text. Lane numbers correspond to the order of the collected fractions. A. Western blot using anti-FITC antibody followed by alkaline phosphatase-conjugated secondary antibody. B. Silver stain. Abbreviations: s = molecular weight standard, b = blank lane.

corresponding to the fluorescent fractions isolated from FPR expressing cells were collected (Fig. 7).

### **Western Blot and Silver Stain Analyses**

To further analyze whether the fluorescent-labeled HPLC fractions contained FPR, the selected fractions were subjected to SDS-PAGE and the separated proteins were analyzed by Western blot and silver stain. The anti-FITC antiserum was utilized to detect the photocrosslinked FPR which appeared as a broad band ~65 kDa (Fig. 8A, lanes 4-7) with the highest intensity corresponding to the fractions representing the fluorescence peak (Fig. 6A). The silver stain also revealed a band at ~65 kDa in the earlier fractions (Fig. 8B, lanes 4-7) with no major contaminating proteins. The later fractions contained a lower molecular weight protein contamination (Fig. 8B, lanes 8 and 9). Silver stain results supported by the immunoblot strongly indicated that we had been able to obtain pure FPR from the CHO cells.

### **Discussion**

CHO cells are frequently used for expression of human G protein-coupled receptors. Samama *et al.* (51) used CHO cells for expression of  $\beta_2$ -adrenergic receptor and were able to obtain cell lines stably expressing

wild-type and mutant receptors. More recently, Fiore *et al.* (19) expressed functional FPR, as well as FPR-related lipoxin A<sub>4</sub> receptor in CHO cells. In addition to expression in CHO cells, functional FPR has been expressed in several different cell lines, such as monkey COS 7 cells (43), mouse L cell fibroblasts (49), and human kidney 293 cells (15). The study of signal transduction does not require isolated receptors and, in most cases, intact expressing cells or their membrane preparations are sufficient to perform the experiments. Several methods of partial purification of FPR from prepared membranes have been reported. Jesaitis *et al.* (27) obtained partially purified, photoaffinity labeled FPR by sedimentation of OG solubilized membranes of neutrophils on a sucrose density gradient. Schreiber *et al.* (56) immunoprecipitated the OG solubilized FPR, from the FPR-expressing, transfected mouse fibroblast (TX2 cells) membranes, with anti-FPR antibody. Immunoprecipitation was also employed by Quehenberger *et al.* (50) to isolate FPR from OG solubilized membranes of Sf9 cells infected with recombinant baculovirus.

In this study, we used CHO cells expressing FPR to produce receptor. The photocrosslinked FPR was successfully extracted from the membranes of CHO cells with OG and purified by HPLC. Our Western blot and silver stain analyses indicated that the HPLC peak fluorescence

fractions contained purified FPR. The relative molecular weight of FPR expressed in CHO cells was comparable to the reported size of FPR partially extracted from the membranes of neutrophils (41). This suggests that the glycosylation processes in the two cells lines are similar. To our knowledge, this is the first report of FPR purification. The purified receptor will be used in further experiments.

**CHAPTER 4****FORMYL PEPTIDE RECEPTOR SCREENING WITH  
PHAGE-DISPLAY PEPTIDE LIBRARY****Introduction**

The functions of FPR, as well as many other receptors, are mediated by protein-protein interactions. These interactions involve specific amino acid sequences displayed on the interacting molecules. The identification of the protein-protein binding sites has been made possible by the use of random peptide libraries, in particular phage-display peptide libraries. Up to billions of unique, short peptide sequences can be displayed on the surface of filamentous phage. Through various affinity purification methods, phage displaying peptides of interest can be isolated from the library and propagated in bacterial cells. The single-stranded DNA (ssDNA) of F-specific bacteriophages, such as M13, encode two major components of the viral coat: protein III and protein VIII (37). To construct the library, the oligonucleotides encoding the random amino acid sequences are ligated to the DNA encoding one or the other of the coat proteins. The library used in this study encodes the random amino acid sequences on three to five copies

of protein III, which are located at one end of the virion (for review see (10)). This protein is necessary for the infection, during which it attaches to the F pilus of the bacterium allowing the insertion of the viral genome. Through the cell's molecular machinery, the complementary DNA strand is produced forming a viral double-stranded DNA (dsDNA), referred to as replicative form (RF). The RF is required for mRNA transcription and ssDNA synthesis. The virions assemble at the periplasmic space and extrude from the cell without killing the bacterium (37). The single-stranded genome of virions facilitates the DNA sequencing of the affinity-selected, amplified phage. The amino acid sequences that are obtained from the DNA sequences may form a consensus sequence of a molecule that interacts with the purified target protein. There are several nucleotide, amino acid, as well as protein sequence databases, which can be helpful in the search and the identification of a molecule containing the consensus sequence.

Phage-display peptide libraries have been successfully used in the identification of interacting sites between proteins and antibodies (12,57), as well as the identification of peptides that bind to proteins and play a role in mediating their functions (14). Jellis *et al.* (26) constructed a 20-amino acid library (PDL-20) used to identify the recognition sequence of a monoclonal antibody (mAb) raised against a HIV-1 isotype MN envelope

protein. The recognition sequence consisted of 4 amino acid residues located at different positions within the random sequence of selected clones from PDL-20. More recently, Burritt *et al.* (11) constructed a nonapeptide library. This library, in conjunction with a hexapeptide library (57), was employed to map epitopes of mAbs specific for the cytochrome *b* components, p22<sup>phox</sup> and gp91<sup>phox</sup>. The identified consensus peptide sequences were nearly identical to the corresponding regions of primary structure sequence of cytochrome *b*. The same nonapeptide and hexapeptide libraries were employed by DeLeo *et al.* (13) for identification of five potential sites of interaction on cytochrome *b* subunits with a cytosolic component of NADPH oxidase, p47<sup>phox</sup>. Synthetic peptides that mimicked two identified sequences found on gp91<sup>phox</sup> cytochrome *b* subunit, were able to inhibit NADPH oxidase activity when added prior to assembly of the oxidase, demonstrating the biological significance of the identified sequences.

In this study, the phage-display peptide libraries were used to select phage that bind FPR. The affinity purification of the phage was performed using two different methods. The first method involved column screening of the library using purified FPR coupled to CNBr-activated Sepharose 4B beads. The goal with this method was to identify G protein sequences involved in interactions with FPR and perhaps to

discover novel FPR-interacting proteins. The second method of library screening involved intact CHO-FPR cells attached to tissue culture plate. A similar method was employed by Dr. Pincus for the selection of phage that bind to HIV-infected H9 cells (personal communication). The goal of our phage library screening with intact cells was to identify nonformylated amino acid sequences that bind FPR.

### **Materials and Methods**

#### **Phage Peptide Library Screening Using FPR Coupled to CNBr- Activated Sephacrose 4B Beads**

HPLC fractions containing a total of ~5 $\mu$ g of FPR were added to 100 mg of beads washed with 1 mM HCl. The beads were then incubated for 24 h at 4 $^{\circ}$ C under rotation and 1% OG and 1 M Tris at pH 8.0 were added to block the remaining reactive groups on the beads. The FPR-coupled beads were stored in glycerol and washed with phage buffer (50 nM Tris-Cl pH 7.5, 150 mM NaCl, 0.5% Tween 20, 1 mg/ml BSA) prior to phage selections. For each selection, approximately one third of the beads was placed in a 5 ml plastic column barrel (Evergreen) and incubated 24 h at 4 $^{\circ}$ C with 75  $\mu$ l of nonapeptide phage library ( $1 \times 10^{12}$  phage) in phage buffer. After incubation, the beads were washed with 50

ml of phage buffer and phage were eluted with eluting buffer (0.1 M glycine, pH 2.2) immediately followed by addition of 2 M Trizma base (to final molarity of 150 mM) to neutralize the pH environment of the phage. The eluate was collected for titration of the phage and further selection.

### **Amplification of Selected Phage**

The eluted phage were amplified by incubating with a fresh culture of K91 *E. coli* for 15 min before mixing with 35 ml of soft agar and spreading on a sterile 9 x 24-inch glass plate with LB. The plate was incubated 24 h in 37°C and phage were lifted from the bacterial lawn by gently rotating with TBS (50 mM Tris-Cl, pH 7.5, 150 mM NaCl) for 3 h at RT. The collected phage were precipitated by incubation with 0.15 volumes of 16.7 % PEG/3.3 M NaCl on ice for 24 hours. The precipitated phage were sedimented by centrifugation (25 min, 4°C, 10,000 rpm, SS34 rotor), resuspended in TBS and microcentrifuged for 3 min at 12,000 rpm. The supernatant was incubated for 1 h on ice with 0.15 volumes of 16.7 % PEG/3.3 M NaCl and microcentrifuged for 2 min. The pellet was resuspended in 300 µl of TBS. The amplified phage was used for subsequent rounds of selections and phage amplifications. Control phage were obtained by two methods. First method involved phage selection on uncoupled (blank) CNBr-activated Sepharose 4B beads,

second method involved selection on beads reacted with HPLC fractions of CHO-WT extracts corresponding to fluorescence fractions of CHO-FPR extracts. The selections and amplifications of control phage were performed in the same manner as where phage were selected with FPR. The phage titer was assessed after each round of selection. Briefly, 100  $\mu$ l of phage serial dilutions were mixed with 250  $\mu$ l of fresh K91 culture, 4 ml of soft agar and plated on LB plates and incubated in 37°C for 24 h. The resulting plaques were counted and the amount of phage/ml was determined.

#### **Phage Library Screening Using CHO-FPR Cells**

Mixed library, consisting of 50  $\mu$ l of linear 9-mer ( $6.6 \times 10^{11}$  phage) and 50  $\mu$ l of circular 10-mer ( $1 \times 10^{12}$  phage) libraries, diluted in TBS/BSA, was preabsorbed for 1 h at 37°C on a confluent 15 cm plate of CHO-WT cells. The preabsorption was repeated twice, each time on a fresh plate of CHO-WT cells. The phage bound to cells after the third preabsorption were used as control phage. The unbound phage were selected on Na-butyrate induced CHO-FPR cells by incubating 1 h at 37°C. After the incubation the cells were washed 5 times with TBS and lysed with 1% NP40 for 10 min. The collected lysate was mixed with soft agar and fresh culture of K91 *E. coli* and spread on LB (9 x 24-inch)

plate. The plate was incubated overnight at 37°C and amplified phage were collected and precipitated as described above. The amplified phage were used for the next round of selection. Total of four rounds of selections and amplifications were performed.

### **Flow Cytometry of Selected Phage Bound to CHO Cells**

CHO-FPR and CHO-WT cells were removed from the tissue culture plates with 1 mM EDTA in PBS and resuspended in cold PBS++ (Ca<sup>2+</sup>/Mg<sup>2+</sup>) supplemented with 5% FBS and aliquoted into eppendorf tubes. Various concentrations of selected and control phage were incubated with the cells for 1 h at 4°C under rotation. The phage to receptor ratios used were 10:1, 1:1 and 1:10. The average number of receptors in CHO-FPR cells had been previously determined from binding of formylated, fluorescein-conjugated peptide to the cells using fluorescent beads as a standard (Flow Cytometry Standards Corporation, San Juan, PR). Approximately  $1 \times 10^6$  receptors were expressed on Na-butyrate induced CHO-FPR cell. After the incubation the cells were microcentrifuged (2000 rpm, 3 min), washed one time with 1 ml PBS++, microcentrifuged again and rabbit anti-M13 serum was added at proper dilution (1:10,000). The dilution was previously determined by examining the background levels produced by different serum

concentrations. After incubation and wash, FITC-conjugated goat anti-rabbit antibody was added (1:200). Following the last incubation, the cells were washed and transferred into FACScan tubes. Cell-associated mean fluorescence was recorded with flow cytometer and analyzed using the GraphPad Prism computer program.

### **Sequencing of Selected Phage Clones**

Single isolated plaques were picked with sterile pipet tips, inoculated into 2 ml of 2 x YT (tryptone, yeast extract, NaCl) containing 75 µg/ml of kanamycin and incubated in 37°C overnight with vigorous shaking. After the incubation, the bacteria were sedimented by microcentrifugation at 12,000 rpm for 5 min and 138 µl of 40% PEG-8000 along with 138 µl of 5M NaOAC pH 7.0 was added to 1.25 ml of supernatant and placed on ice for 24 h. The precipitated phage were microcentrifuged at 12,000 rpm for 15 min and the pellet was resuspended in 30 µl of TE. The purified phage were then immersed in boiling water bath for 5 min to release phage DNA. The sequencing reactions using isolated phage DNA were performed following the directions of the Sequenase version 2.0 kit (U. S. Biochemical Corp.) using the sequencing primer 5'-gttttgtcgtctttccagacg-3', which allowed the synthesis of the random oligonucleotide region of the phage. The

sequences were read from the gel image obtained using a Molecular Dynamics model 400E PhosphorImager.

## Results

### **Affinity Purification of Phage Using FPR-Coupled CNBr- Activated Sepharose 4B**

To select FPR-binding phage from nonapeptide phage library, the HPLC purified receptor was immobilized on CNBr-activated Sepharose 4B beads and the library was incubated with the beads. Phage that bound to FPR were eluted from the column with low pH, amplified, used in two more rounds of selection on previously unused FPR-coupled beads, and sequenced. With each round of selection a similar increase in titers of the phage selected on FPR-coupled beads, and the phage selected on blank beads, was observed (Table 1). Moreover, there was not a significant difference between the FPR-selected and control phage titers obtained after the final selections of two experiments utilizing two types of control beads; the blank beads and the beads that were reacted with HPLC fractions of CHO-WT extracts corresponding to fluorescence fractions of CHO-FPR cell extracts. These observations suggest that the selection of the phage was not FPR specific. The obtained random region sequences of the FPR-selected phage revealed a consensus sequence

Table 1. Phage titers (phage/ml) after each round of selections.

Selection #1				Selection #2			
FPR <sup>1</sup>		Blank <sup>2</sup>		FPR <sup>1</sup>		WT <sup>3</sup>	
Wash	Eluate	Wash	Eluate	Wash	Eluate	Wash	Eluate
<b>Round 1</b>							
3 x 10 <sup>7</sup>	1 x 10 <sup>6</sup>	9 x 10 <sup>8</sup>	2 x 10 <sup>6</sup>	3.6 x 10 <sup>12</sup>	3.5 x 10 <sup>12</sup>	ND	2 x 10 <sup>12</sup>
<b>Round 2</b>							
1.3 x 10 <sup>10</sup>	7.2 x 10 <sup>9</sup>	1.4 x 10 <sup>10</sup>	5.7 x 10 <sup>8</sup>	1 x 10 <sup>10</sup>	4 x 10 <sup>8</sup>	1 x 10 <sup>10</sup>	5.4 x 10 <sup>8</sup>
<b>Round 3</b>							
2 x 10 <sup>10</sup>	1.5 x 10 <sup>10</sup>	1.4 x 10 <sup>10</sup>	1 x 10 <sup>10</sup>	1 x 10 <sup>11</sup>	6 x 10 <sup>11</sup>	6.5 x 10 <sup>10</sup>	1 x 10 <sup>10</sup>

<sup>1</sup>Selection carried out using FPR-coupled CNBr-activated Sepharose 4B beads.

<sup>2</sup>Selection carried out using blank CNBr-activated Sepharose 4B beads.

<sup>3</sup>Selection carried out using HPLC fractions of CHO-WT membrane extracts coupled to CNBr-activated Sepharose 4B beads. The fractions corresponded to HPLC elution profile of FPR.

Table 2. Random region amino acid sequences of selected phage. Amino acids are in single letter code.

Selection	FPR-selected phage	Control phage
#1 <sup>a</sup>	W H L	S W F G S G F K G
	W H M G N G K P W	M N A P R Q G S A
	W H L R K P V	A W A
	W H L R G W G P A	A Y R H S F K N L
	W H L R G G G T P	S T L
		T Y A W H L R T Q
#2 <sup>b</sup>	H G H H P H E A W	T N P D E A R R K
	W H L R K P V	T H L K G F K G S
	W H L K	W H R I P X X X X
	W H K G R P A G	S H R F H Y S H Q
	W H Q K F L Y K N	W H K X P X M X S
	H H L R I V Q R G	N H L K Y R L N Q
#3 <sup>c</sup>	T A L H G N	V V V N K N F T L
	X R V T L G R P E L	Q W A P V Q N L V R
	L G A A	K W
	I L F S	V I A T G G M V S
	L S R V G G V T P P	Q S F R Y A G T A
	S I T K S L F T G	V L I D T G
	Y T V Q K V K N T	Q R G S V Q E Y L T
	V N I Y G P H V V	
	M P E N F K H S P	
	A A G E Q M T H R	
	D L T R L H R K L	
	F R S P	
	L R V V	
	L P L N L F D	
X P M E X P S T G V		

<sup>a</sup>Selection carried out using FPR-coupled CNBr-activated Sepharose 4B beads and blank beads for control selection.

<sup>b</sup>Selection carried out using FPR-coupled CNBr-activated Sepharose 4B beads and as a control, beads coupled with HPLC fractions of CHO-WT corresponding to the elution profile of FPR.

<sup>c</sup>Selection carried out using intact CHO-FPR cells and intact CHO-WT cells as a control.

WHLR (Table 2). Although this sequence appeared in two sets of experiments (notice the same WHLRKPV sequence selected in two different experiments), it also appeared in the control phage but with lower frequency when the blank beads were used in the control phage selection. This could suggest that the WHLR expressing phage were specific for a contaminant, which may have eluted from the gel filtration column along with FPR. The presence of the WHLR sequence in the control phage eluted from the blank beads can be explained by the fact that, although fresh blank beads were used for each new round of selection, the phage used were the phage eluted from the FPR-coupled beads in the previous selection.

### **Phage Selection on CHO Cells Expressing FPR**

In order to identify phage that bind to intact cells, mixed linear 9-mer and circular 10-mer libraries were selected on tissue culture plates containing near confluent monolayers of CHO-FPR and CHO-WT cells. The cells with the bound phage were lysed and the lysates were used in phage amplification. A total of four selections and four amplifications were performed and the selected phage were analyzed for cell binding affinity on FACScan before sequencing. FACScan results revealed increased binding of FPR-selected phage to CHO cells expressing FPR at

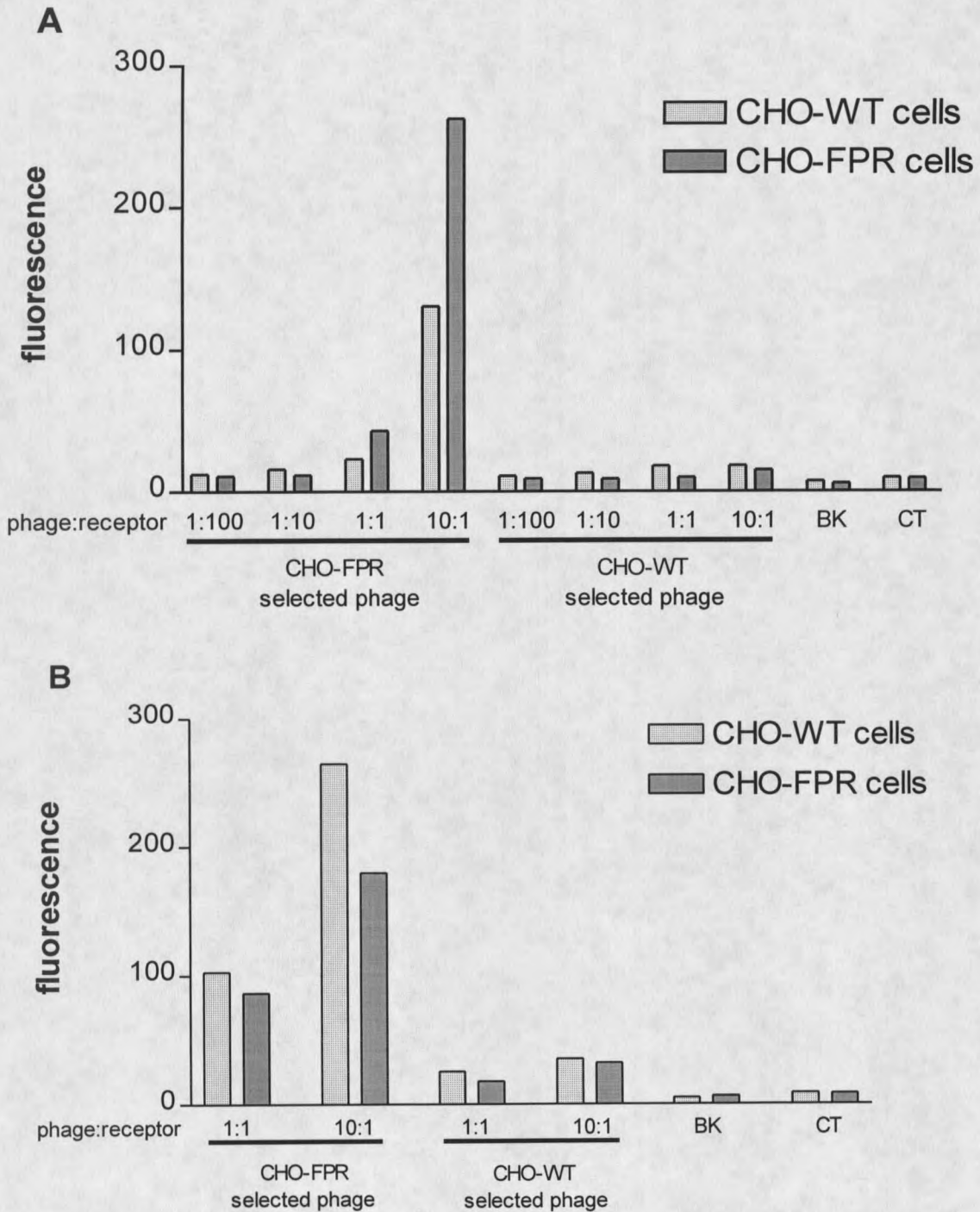


Fig. 9. Flow cytometry analysis of binding of selected phage to CHO-FPR and CHO-WT cells. Intact cells were used in the selection of phage peptide library. A. Only the CHO-FPR cells were induced with Nabutyrate to stimulate increased FPR production. B. Both, the CHO-FPR and the CHO-WT cells were induced.

1:1 phage to receptor ratio, as compared to CHO-WT cells (Fig. 9A). The same trend but to a lesser degree was observed with CHO-WT-selected phage. However, when the FACScan was repeated using CHO-WT cells induced with Na-butyrate, the binding of phage selected with CHO-FPR was higher to CHO-WT cells than to cells expressing FPR (Fig. 9B). This suggests that the phage may have been selected on surface molecules expressed by butyrate-induced cells. The sequences obtained from selected phage did not reveal any strong consensus sequence but several common motifs were observed. The TVQ sequence was selected on CHO-FPR cells as well as on the FPR-coupled beads. The LF and the RV motifs appeared in three out of 15 sequences.

### **Discussion**

The use of phage-display peptide libraries has been successful in identification of peptide binding sites of various proteins. Here, we have used nonapeptide and decapeptide libraries to select phage that bind FPR. The results of phage selections using the FPR-coupled CNBr-activated Sepharose beads revealed a strong consensus sequence, WHLR, but the significance of this finding is unclear because this sequence was also observed in control phage. More experiments need to be done to determine the source of selection of the frequently appearing WHLR

sequence in both the FPR-selected phage and the control phage. The modification of this phage selection method could lead to more successful results. For example, if the phage expressing WHLR in their random region seemed to be selected on the Sepharose beads, maybe the use of a different matrix would allow for more FPR-specific selection and elimination of Sepharose-binding phage. If, on the other hand, the WHLR selection resulted from the contaminant which eluted in the same fraction as the HPLC purified FPR, perhaps a preabsorption of phage on CHO-WT coupled beads would eliminate the contaminant-binding phage. In addition to the coupling to CNBr-activated Sepharose beads, the purified receptor can be utilized in other methods of phage selection, such as biopanning.

Besides the WHLR sequence, the selection of LF motif is interesting as it resembles  $\phi$ MLF. Furthermore, a truncated phage peptide, ILFS, is similar to CHO-ILF-OH, that along with several other peptides resembling  $\phi$ MLF were analyzed for their ability to induce lysosomal enzyme release and chemotactic activities (20). The  $\phi$ ILF peptide was shown to be biologically more active than most of the other peptides. A modification of phage selection method involving the use of high concentration of  $\phi$ MLF (mM range) in eluting the phage could result in

the identification of additional peptides that bind to the ligand binding site.

The selection of phage using intact cells is a novel and promising approach. In our experiments the use of different cells in the selection produced phage with different binding profiles to those cells. However, the FACScan analysis of phage bound to Na-butyrate induced cells gave unexpected results, suggesting that the selected phage may not have been specific for FPR. The use of intact cells in phage peptide library screening was also employed by L. Mazzucchelli, MD (personal communication) to identify high affinity binding peptides to PMNs. The selected phage were specific for neutrophils, as demonstrated by increased binding to PMNs in comparison to peripheral blood lymphocytes, monocytic cells, as well as human epithelial, endothelial and fibroblast cell lines. In an effort to examine the specificity of the phage isolated using intact CHO-FPR cells, we analyzed the binding by flow cytometry. This allowed us to further select the phage populations or individual clones that appeared to have the highest binding affinities toward FPR in intact CHO cells. This approach eliminated the sequencing of phage that may have bound unspecifically to the CHO cells. In summary, although we were unsuccessful in the identification of FPR-binding peptides or proteins, during the course of this work, we

were able to establish conditions and methods for future more extensive library screening.

## CHAPTER 5

### ANALYSIS OF CYTOPLASMIC TAIL DELETION MUTANTS OF FORMYL PEPTIDE RECEPTOR IN CHO CELLS

#### Introduction

The advancement in the recombinant DNA techniques has led to the development of new, powerful tools for studying functions of gene products. One of those tools, commonly used in the analysis of the structure-function relationships of various proteins, is *in vitro* mutagenesis. It is referred to as "reverse genetics", as it allows us to change the gene DNA sequence and then analyze the gene's function (63). Site-specific mutagenesis has been used extensively to study the structure-function relationship of FPR in our laboratory (38) and other laboratories (46,48). In this study, we examined the role of the cytoplasmic tail in cell responses mediated by FPR. The cytoplasmic tail was of interest because it has been previously shown in two independent studies that the middle region of the tail may interact with G protein (7,55). Oligonucleotide-directed mutagenesis was carried out to produce two cytoplasmic tail deletions of FPR, CT9 and CT29. The stop codons were inserted at L<sup>316</sup> (CT9) and T<sup>336</sup> (CT29) positions of the FPR cDNA

sequence (Fig. 10) resulting in the truncated carboxyl-terminal tails. This chapter describes the analysis of the deletion mutants to provide insight into the importance of the deleted sequences in signal transduction and chemotaxis by CHO cells that express FPR.

## **Materials and Methods**

### **Oligonucleotide-Directed Mutagenesis of FPR**

The FPR cDNA sequence was inserted into Bluescript SK<sup>+</sup> and transfected into a strain of *E. coli* (CJ 236), which lacks two enzymes, dUTPase and uracil N-glycosylase, necessary for proper uracil processing. The infection of the bacteria with M13K07 helper phage resulted in the production of uracil-containing single stranded (ss) DNA (U-DNA). The helper phage allowed the U-DNA to be properly packaged into phage particle (35). The U-DNA was isolated and mutagenic oligonucleotides containing stop codons (+10 nucleotides complementary in both directions) were annealed and complementary strand was synthesized with T4 DNA polymerase. The double-stranded DNA mixture was transfected into an *E. coli* strain with normal N-glycosylase activity, which allowed the U-containing strand, which does not contain the mutation, to be destroyed. The mutations were verified by sequencing.

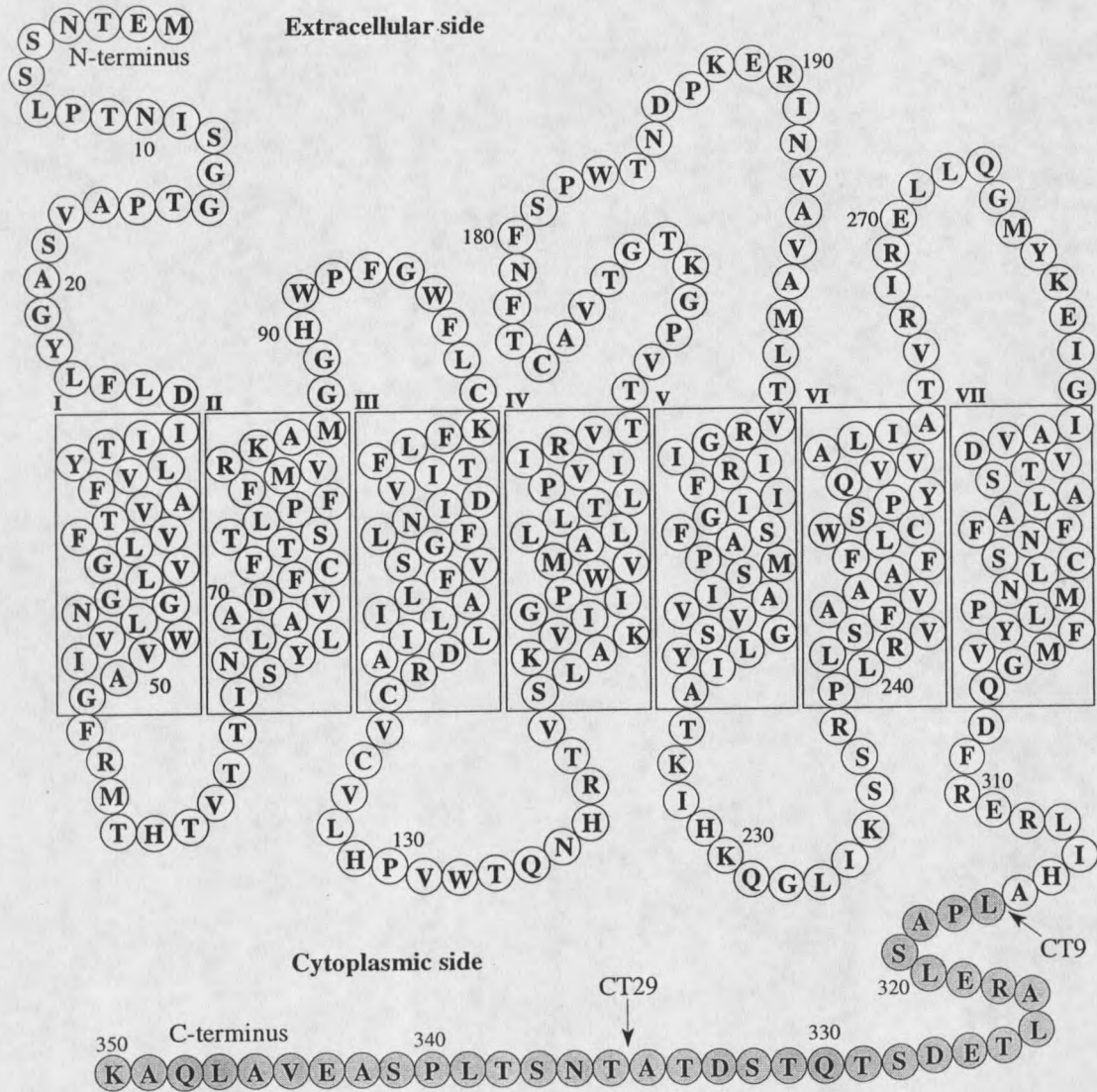


Fig. 10. Schematic representation of FPR based on the Baldwin model (2). Shaded area represents the deleted region. The stop codons resulting in CT9 and CT29 deletions were inserted at L<sup>316</sup> and T<sup>336</sup>, respectively.

### **Ligation and Transfection**

The cDNA was ligated into the *EcoRI* site of pBGSA. pBGSA is a vector designed for stable expression of exogenous genes in mammalian cells (62). It confers the Geneticin (G418) resistance to transfected cells. The ligation mixture was transfected into *E. coli* and individual clones were analyzed to identify expression vectors containing the insert in right orientation. The vector was transfected into wild type CHO cells by lipofection (18). After a week of culture, twelve G418-resistant colonies of each mutant were picked and plated into separate wells for analysis of expression levels.

### **FACScan Analysis of FPR Expression of Each Mutant Clone**

The cells were rinsed in PBS and incubated in PBS containing 1.1 mM EDTA and  $\sim 4 \mu\text{M}$  trypsin. Cells were allowed to round up and were removed from the plates in cold PBS ++ with 5% FBS and 1 ml aliquots were placed in FACScan tubes on ice. 10 nM of fNleLFNleYK-fluorescein was added to each tube and incubated for 1 h. The mean fluorescence of the examined clones was compared to the mean fluorescence of CHO-WT and CHO-FPR cells. The average number of mutant receptors per cell was calculated from two (CT9 and CT29) best expressing cell lines by

comparing them with the mean fluorescence of CHO-FPR. The background fluorescence was determined using CHO-WT cells.

### **Analysis of Mutant FPR Expression by Immunofluorescence**

The cells were grown on glass coverslips until about 70-80% confluent, rinsed with cold PBS and incubated 15 min on ice with 200 nM  $\mu$ MBpaFYK-fluorescein peptide. Next, the cells were subjected to UV light for 10 min to allow for photocrosslinking of the peptide to the receptor. Gently rinsed coverslips were mounted on slides and observed with fluorescence microscope.

### **Intracellular Calcium Release Assay**

The cells were washed in PBS, incubated with 1 mM EDTA, gently scraped off the plates and resuspended in PBS++ with 5% FBS and 2  $\mu$ M Fura-2AM. The cells were incubated in 37°C for 40 min, sedimented (Beckman 1000 rpm for 5 min), and resuspended in PBS++ with 5% FBS. Calcium released by the cells was detected by fluorometer. The measurements were obtained under constant stirring while adding 1  $\mu$ M  $\mu$ MLF at 50 s, 1  $\mu$ M ATP at 100 s, 0.2 % TX-100 at 150 s, and 30 mM EGTA at 200 s. The fluorometer data was analyzed with Felix Software (Photon Technology International, So. Brunswick, NJ).

**Chemotaxis assay**

The cells were removed from the plates with trypsin-EDTA, resuspended in medium with 6 mM Na-butyrate and incubated 1 h at 37°C. Next, the cells were resuspended in medium without serum with 6 mM Na-butyrate and plated on 8 µm pore Transwell inserts at densities of  $3 \times 10^5$  cells per insert. The following concentrations of chemoattractants were added into lower wells: 1 nM fMLF, 10 nM fMLF, 100 nM fMLF and 20 µg/ml fibronectin (FN). The inserts with the cells were placed in the wells and incubated 4 h at 37°C. After the incubation, the cells that had not migrated through the inserts were removed with cotton swabs. The inserts were placed in wells containing 2.5% PFA and fixed overnight. Next, the inserts were placed in Hematoxylin Gill's stain for 30 min and washed with PBS. Stained filters were removed from the inserts, placed on slides and observed under microscope. The degree of chemotaxis was determined from the average surface area of migrated cells obtained from six different 40x fields examined by an image analysis system (Imaging Research M4 True Color Image Analysis System, Imaging Research, St. Catherines, Ontario).

## Results

### Isolation of Highest Expressing CHO Clones

Each mutant clone was examined by flow cytometry for its ability to bind the fNleLFNleYK-fluorescein ligand. The clones that exhibited the highest mean fluorescence were isolated and the average numbers of CT9 and CT29 mutant receptors were estimated to be  $1.08 \times 10^4$  and  $9.5 \times 10^3$  per cell, respectively. The numbers of receptors per cell were based on the calculated number of receptors on CHO cells expressing WT FPR, assuming that the ligand binding affinity of the mutants equal that of WT FPR. The selected clones were subcultured and used in further experiments.

### Release of Intracellular Calcium by the Cells Expressing Mutant FPRs

To examine the coupling of mutant FPR to G protein, fMLF stimulated cells were observed for their ability to release calcium from intracellular stores. The cells were incubated with Fura-2AM, which can pass through cell membranes. Upon binding to calcium, Fura-2 exhibits spectral response, which can be utilized in detection of changes in calcium concentration in the cytoplasm. The assay results revealed that

upon stimulation with *f*MLF both mutant cell lines were able to release calcium (Fig. 11), suggesting that there was coupling of G protein to the mutated receptors. However, the high concentration of *f*MLF (1  $\mu$ M) required for calcium mobilization by CT9 and CT29 suggests that there was only partial coupling of G protein to mutant receptors or the receptors exhibited low binding affinity for the ligand. Both mutant cell lines, however, were able to release calcium upon stimulation with ATP, which was used as a control to determine cell signal transduction capabilities.

#### ***f*MLF-Mediated Chemotaxis of CHO Cells Expressing Mutant FPRs**

The CHO cells expressing wild type FPR are able to migrate towards *f*MLF, with the maximal migration at 0.1-1 nM, as determined by Dr. Miettinen (personal communication). To examine whether the cells expressing mutant FPRs were also able to migrate towards chemoattractants, the chemotaxis assay was carried out. The cells were incubated in serum-free medium and allowed to migrate through semipermeable filters towards fibronectin, used as a positive control, and different concentrations of *f*MLF. According to the assay results, CHO cells expressing CT9 migrated towards *f*MLF (Fig. 12) with the maximal migration at 10 nM, but there was no FPR-mediated chemotaxis of CHO

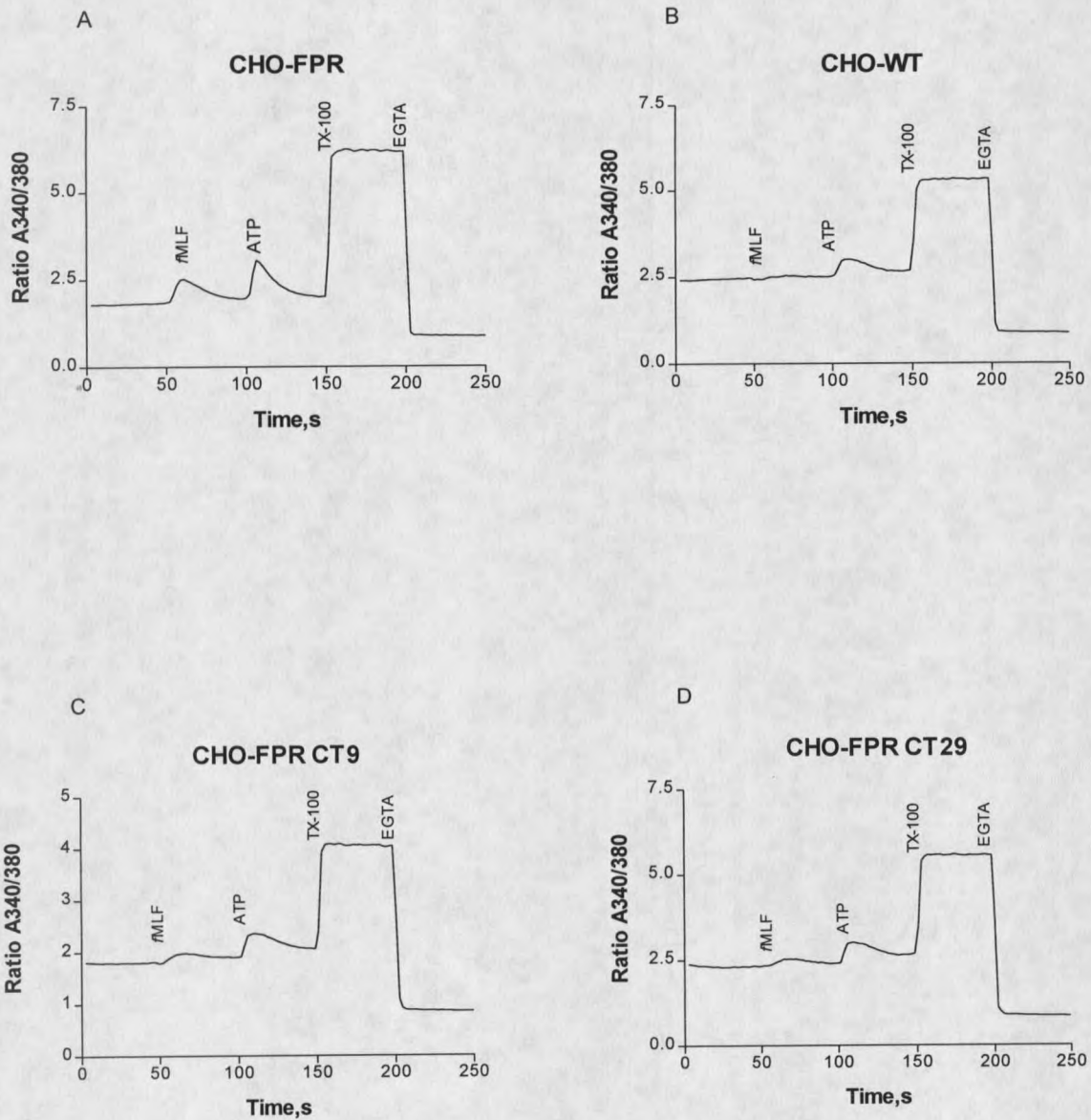


Fig. 11. Release of intracellular calcium by CHO expressing wild-type FPR (A), untransfected CHO cells (B), and the CHO cells expressing the cytoplasmic deletion mutants, CT9 (C) and CT29 (D). fMLF was added to a final concentration of 1  $\mu$ M, ATP to 1  $\mu$ M, TX-100 to 0.2%, and EGTA to 30 mM.

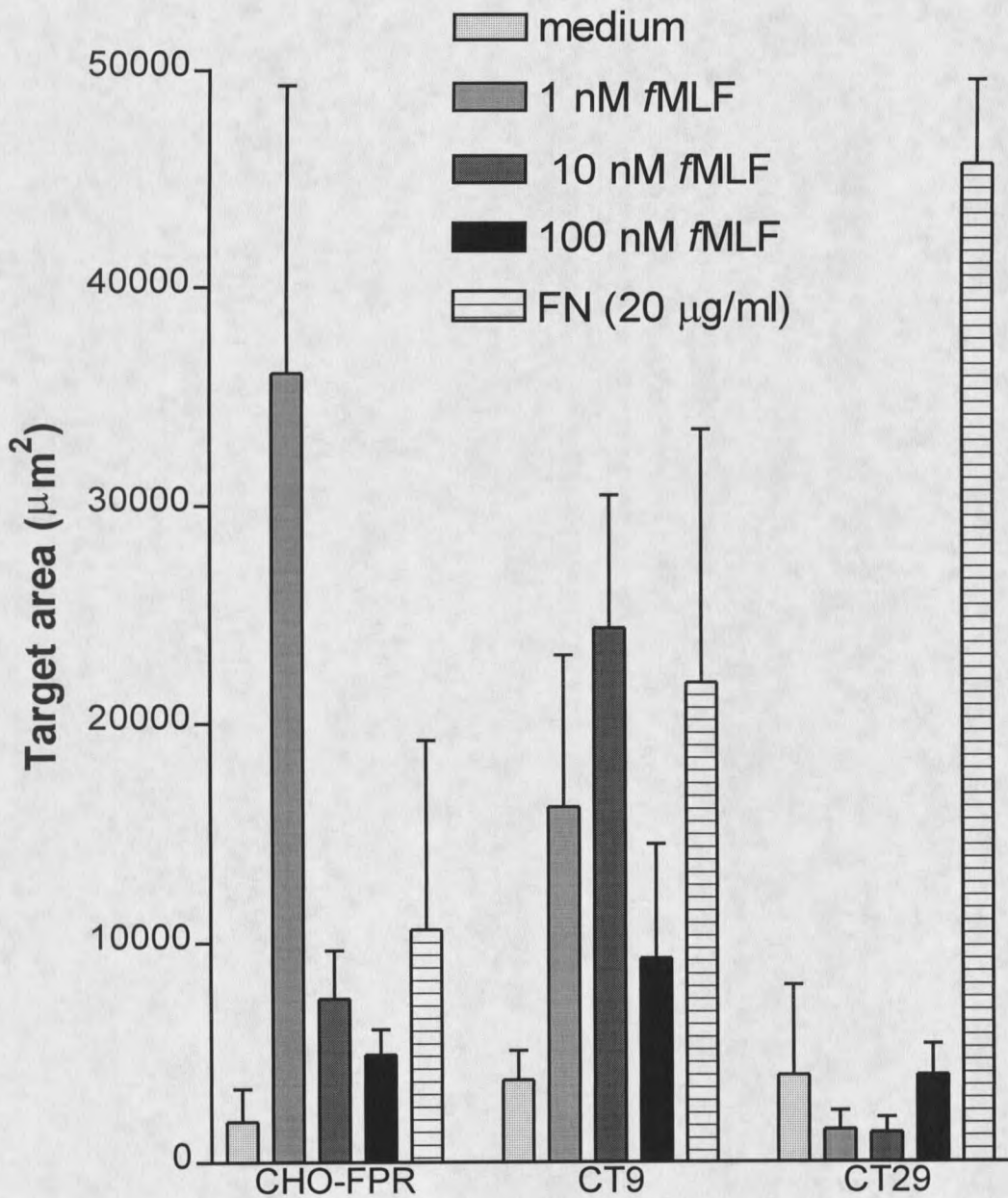


Fig. 12. Chemotaxis assay of CHO-FPR and the cytoplasmic deletion mutants, CT9 and CT29. The cells were allowed to migrate towards different concentrations of fMLF or 20  $\mu\text{g}/\text{ml}$  fibronectin (FN), as marked. Bars represent standard deviation calculated from six different random fields of a semipermeable filter.

cells expressing CT29. Since CT9 had a larger deletion than CT29, these results were somewhat unexpected and could be due to the low expression of mutant FPRs. The low expression could also explain the inability to effectively carry out the GTP $\gamma$ S assay on the mutants, the inability to determine  $K_{ds}$  of mutant FPRs, as well as the difficulty visualizing photoaffinity labeled cells. The results from the chemotaxis assay suggest that the deleted regions of FPR carboxyl terminus may play a role but are not absolutely necessary in mediating migration of cells expressing the mutant FPRs towards  $\beta$ MLF.

### Discussion

In this chapter, we described the functional analysis of two cytoplasmic tail deletion mutants of FPR. The stop codons were inserted in positions L<sup>316</sup> and T<sup>336</sup> to produce receptors with 9- and 29-amino acid long cytoplasmic tails, respectively. It has been demonstrated by Bommakanti *et al.* that a synthetic peptide corresponding to the <sup>322</sup>RALTEDSTQTS<sup>336</sup> sequence of FPR competed with neutrophil receptor for binding to bovine G protein, suggesting that the carboxyl-terminal tail of FPR was involved in coupling to G protein (7). Since the cellular responses to  $\beta$ MLF observed with the mutants were not of the same magnitude as with the WT FPR, it may suggest several possibilities:

1. The cytoplasmic tail may be important for G protein coupling but other regions of the receptor may also participate in this interaction in a fashion similar to ligand binding to multiple different residues. This possibility is supported by results by Schreiber *et al.* (55) and Bommakanti *et al.* (6). Through competitive ELISA, as well as FPR-G protein reconstitution assays, Schreiber *et al.* provided evidence that, in addition to the carboxyl-terminal tail, the FPR second intracellular loop also contained a contact site with G protein (55). More recently, Bommakanti *et al.* examined thirteen synthetic FPR peptides for their ability to disrupt FPR-G protein complexes and concluded that all three cytoplasmic loops and parts of cytoplasmic tail are involved in the physical interaction between the receptor and G protein (6).

2. It is possible that the poor response of the mutants was due to a low expression level of the mutant receptors. Based on the flow cytometry results of various mutant clones, we were able to estimate the number of receptors per cell at about  $1 \times 10^4$ , a 100-fold decrease in expression as compared to the WT receptors.

3. Finally, the mutations may have resulted in a structural change in the receptor altering the ligand binding affinity. Through analysis of FPR chimeras, Perez *et al.* have demonstrated that the carboxyl-terminal tail was capable of modulating receptor affinity for the ligand (43). Perhaps the cytoplasmic tail

deletions resulted in receptors with very low ligand binding affinity, which could explain the difficulty in obtaining the dissociation constants of the mutant receptors. Additional site-directed mutagenesis studies might allow the determination of the residues that play critical role in the FPR-G protein interaction. Furthermore, phage peptide library screening using peptides that mimic the FPR sequences could lead to the identification of specific residues involved in this interaction.

## CHAPTER 6

### CONCLUSIONS

1. The expression of formyl peptide receptor (FPR) by Sf9 insect cells results in a receptor with decreased ligand binding affinity ( $K_d=70$  nM), as compared to FPR in neutrophils ( $K_d=3$  nM).

2. Photocrosslinked FPR expressed in Chinese hamster ovary (CHO) cells can be effectively extracted from cell membranes with octylglucoside and purified by high performance liquid chromatography.

3. Phage-display peptide library selection using either FPR-coupled cyanogen bromide-activated Sepharose 4B beads or intact CHO cells expressing FPR produced inconclusive results. Modifications of phage library selection methods may allow the identification of peptides that bind to the receptor.

4. Functional analyses of two FPR cytoplasmic tail deletion mutants, CT9 and CT29, revealed partial calcium release and suppressed chemotaxis. This suggests that the cytoplasmic region of FPR is not absolutely necessary for FPR-mediated signal transduction.

## LITERATURE CITED

1. **Alberts B., D. Bray, J. Lewis, M. Raff, K. Roberts, and J. D. Watson.** 1994. Cell signaling. In *Molecular biology of the cell*. 3rd ed. Garland Publishing Inc., New York, p. 721.
2. **Baldwin, J. M.** 1993. The probable arrangement of the helices in G protein-coupled receptors. *EMBO J.* 12:1693.
3. **Becker, E. L., J. C. Kermode, P. H. Naccache, R. Yassin, M. L. Marsh, J. J. Munoz, and R. I. Sha'afi.** 1985. The inhibition of neutrophil granule enzyme secretion and chemotaxis by pertussis toxin. *J. Cell Biol.* 100:1641.
4. **Birnbaumer, L.** 1990. Transduction of receptor signal into modulation of effector activity by G proteins: the first 20 years or so... *FASEB J.* 4:3178.
5. **Bokoch, G. M., and A. G. Gilman.** 1984. Inhibition of receptor-mediated release of arachidonic acid by pertussis toxin. *Cell* 39:301.
6. **Bommakanti, R. K., E. A. Dratz, D. W. Siemsen, and A. J. Jesaitis.** 1995. Extensive contact between Gi2 and N-formyl peptide receptor of human neutrophil: mapping of binding sites using receptor-mimetic peptides. *Biochemistry* 34:6720.
7. **Bommakanti, R. K., K. N. Klotz, E. A. Dratz, and A. J. Jesaitis.** 1993. A carboxyl-terminal tail peptide of neutrophil chemotactic receptor disrupts its physical complex with G protein. *J. Leukoc. Biol.* 54:572.
8. **Boulay, F., M. Tardiff, L. Brouchon, and P. Vignais.** 1990. The human N-formylpeptide receptor. Characterization of two cDNA isolates and evidence for a new subfamily of G-protein-coupled receptors. *Biochemistry* 29:11123.
9. **Browning, D. D., Z. K. Pan, E. R. Prossnitz, and R. D. Ye.** 1997. Cell type- and developmental stage-specific activation of NF-kappaB by fMet-Leu-Phe in myeloid cells. *J. Biol. Chem.* 272:7995.

10. **Burritt, J. B. and C. W. Bond.** 1996. Review. Filamentous phage display of oligopeptide libraries. *Anal. Biochem.* 238:1.
11. **Burritt, J. B., M. T. Quinn, M. A. Jutila, C. W. Bond, and A. J. Jesaitis.** 1995. Topological mapping of neutrophil cytochrome b epitopes with phage-display libraries. *J. Biol. Chem.* 270:16974.
12. **Cwirla, S. E., E. A. Peters, R. W. Barrett, and W. J. Dower.** 1990. Peptides on phage: a vast library of peptides for identifying ligands. *Proc. Natl. Acad. Sci. USA* 87:6378.
13. **DeLeo, F. R., L. Yu, J. B. Burritt, L. R. Loetterle, C. W. Bond, A. J. Jesaitis, and M. T. Quinn.** 1995. Mapping sites of interaction of p47-phox and flavocytochrome b with random-sequence peptide phage display libraries. *Proc. Natl. Acad. Sci. USA* 92:7110.
14. **Devlin, J. J., L. C. Panganiban, and P. E. Devlin.** 1990. Random peptide libraries: a source of specific protein binding molecules. *Science* 249:404.
15. **Didsbury, J. R., R. J. Uhing, E. Tomhave, C. Gerard, N. Gerard, and R. Snyderman.** 1991. Receptor class desensitization of leukocyte chemoattractant receptors. *Proc. Natl. Acad. Sci. USA* 88:11564.
16. **Engelman, D. M., R. Henderson, A. D. McLachlan, and B. A. Wallace.** 1980. Path of the polypeptide in bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA* 77:2023.
17. **Fay, S. P., R. G. Posner, W. N. Swann, and L. A. Sklar.** 1991. Real-time analysis of the assembly of ligand, receptor, and G protein by quantitative fluorescence flow cytometry. *Biochemistry* 30:5066.
18. **Felgner, P. L., T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrop, G. M. Ringold, and M. Danielsen.** 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. USA* 84:7413.
19. **Fiore, S., J. F. Maddox, H. D. Perez, and C. N. Serhan.** 1994. Identification of a human cDNA encoding a functional high affinity lipoxin A4 receptor. *J. Exp. Med.* 180:253.

20. **Freer, R. J., A. R. Day, J. A. Radding, E. Schiffmann, S. Aswanikumar, H. J. Showell, and E. L. Becker.** 1980. Further studies on the structural requirements for synthetic peptide chemoattractants. *Biochemistry* 19:2404.
21. **Gat, U., E. Nekrasova, D. Lancet, and M. Natchin.** 1994. Olfactory receptor proteins: expression, characterization and partial purification. *Eur. J. Biochem.* 225:1157.
22. **Gorman, C. M. and B. H. Howard.** 1983. Expression of recombinant plasmids in mammalian cells is enhanced by sodium butyrate. *Nucl. Acids Res.* 11:7631.
23. **Greenfield, C., G. Patel, S. Clark, N. Jones, and M. D. Waterfield.** 1988. Expression of the human EGF receptor with ligand-stimulatable kinase activity in insect cells using a baculovirus vector. *EMBO J.* 7:139.
24. **Hsieh, P. and P. W. Robbins.** 1984. Regulation of asparagine-linked oligosaccharide processing. Oligosaccharide processing in *Aedes albopictus* mosquito cells. *J. Biol. Chem.* 259:2375.
25. **Iizuka, M. and K. Fukuda.** 1993. Purification of the bovine nicotinic acetylcholine receptor  $\alpha$ -subunit expressed in baculovirus-infected insect cells. *J. Biochem.* 114:140.
26. **Jellis, C. L., T. J. Cradick, P. Rennert, P. Salinas, J. Boyd, T. Amirault, and G. S. Gray.** 1993. Defining critical residues in the epitope for a HIV-neutralizing monoclonal antibody using phage display and peptide array technologies. *Gene* 137:63.
27. **Jesaitis, A. J., J. O. Tolley, G. M. Bokoch, and R. A. Allen.** 1989. Regulation of chemoattractant receptor interaction with transducing proteins by organizational control in the plasma membrane of human neutrophils. *J. Cell Biol.* 109:2783.
28. **Katada, T. and M. Ui.** 1982. Direct modification of the membrane adenylate cyclase system by islet-activating protein due to ADP-ribosylation of a membrane protein. *Proc. Natl. Acad. Sci. USA* 79:3129.

29. **Klotz, K. N. and A. J. Jesaitis.** 1994. The interaction of N-formyl peptide chemoattractant receptors with the membrane skeleton is energy-dependent. *Cell. Signal.* 6:943.
30. **Koo, C., R. J. Lefkowitz, and R. Snyderman.** 1982. The oligopeptide chemotactic factor receptor on human polymorphonuclear leukocyte membranes exists in two affinity states. *Biochem. Biophys. Res. Commun.* 106:442.
31. **Koo, C., R. J. Lefkowitz, and R. Snyderman.** 1983. Guanine nucleotides modulate the binding affinity of the oligopeptide chemoattractant receptor on human polymorphonuclear leukocytes. *J. Clin. Invest.* 72:748.
32. **Lad, P. M., C. V. Olson, and P. A. Smiley.** 1985. Association of the N-formyl-met-leu-phe receptor in human neutrophils with a GTP-binding protein sensitive to pertussis toxin. *Proc Natl. Acad. Sci. USA* 82:869.
33. **Malech, H. L., J. P. Gardner, D. F. Heiman, and S. A. Rosenzweig.** 1985. Asparagine-linked oligosaccharides on formyl peptide chemotactic receptors of human phagocytic cells. *J. Biol. Chem.* 260:2509.
34. **Marasco, W. A., S. H. Phan, H. Krutzsch, H. J. Showell, D. E. Feltner, R. Nairn, E. L. Becker, and P. A. Ward.** 1984. Purification and identification of formyl-methionyl-leucyl-phenylalanine as the major peptide neutrophil chemotactic factor produced by *Escherichia coli*. *J. Biol. Chem.* 259:5430.
35. **McClary, J., F. Witney, and J. Geisselsoder.** 1989. Efficient site-directed in vitro mutagenesis using phagemid vectors. *Bio-Rad Lab.* 8:1.
36. **McPhail, L. C., D. Qualliotine-Mann, D. E. Agwu, and C. E. McCall.** 1993. Phospholipases and activation of the NADPH oxidase. *Eur. J. Haematol.* 51:294.
37. **Messing, J.** 1993. New M13 vectors for cloning. *Meth. Enzymol.* 101:20.
38. **Miettinen, H. M., J. S. Mills, J. M. Gripenrog, and A. J. Jesaitis.** 1997. The ligand binding domain of the formyl peptide receptor maps in the transmembrane region. *Submitted for publication.*

39. **Moss, J. and M. Vaughan.** 1979. Activation of adenylate cyclase by cholera toxin. *Ann. Rev. Biochem.* 48:581.
40. **Narayan, P., J. Gray, and D. Puett.** 1996. Expression of functional lutropin/choriogonadotropin receptor in the baculovirus system. *Mol. Cell. Endocrin.* 117:95.
41. **Niedel, J., J. Davis, and P. Cuatrecasas.** 1980. Covalent affinity labeling of the formyl peptide chemotactic receptor. *J. Biol. Chem.* 255:7063.
42. **Okajima, F. and M. Ui.** 1984. ADP-ribosylation of the specific membrane protein by islet-activating protein, pertussis toxin, associated with inhibition of a chemotactic peptide-induced arachidonate release in neutrophils. *J. Biol. Chem.* 259:13863.
43. **Perez, H. D., R. Holmes, L. Vilander, R. R. Adams, W. Manzana, D. Jolley, and W. H. Andrews.** 1993. Formyl peptide receptor chimeras define domains involved in ligand binding. *J. Biol. Chem.* 268:2292.
44. **Perez, H. D., E. Kelly, F. Elfman, G. Armitage, and J. Winkler.** 1991. Defective polymorphonuclear leukocyte formyl peptide receptor(s) in juvenile periodontitis. *J. Clin. Invest.* 87:971.
45. **Pillinger, M. H. and S. B. Abramson.** 1995. The neutrophil in rheumatoid arthritis. *Rheum. Dis. Clin. North Am.* 21:691.
46. **Prossnitz, E. R., C. M. Kim, J. L. Benovic, and R. D. Ye.** 1995. Phosphorylation of the N-formyl peptide receptor carboxyl terminus by the G protein-coupled receptor kinase GRK2. *J. Biol. Chem.* 270:1130.
47. **Prossnitz, E. R., O. Quehenberger, C. G. Cochrane, and R. D. Ye.** 1991. Transmembrane signalling by the N-formyl peptide receptor in stably transfected fibroblasts. *Biochem. Biophys. Res. Comm.* 179:471.
48. **Prossnitz, E. R., R. E. Schreiber, G. M. Bokoch, and R. D. Ye.** 1995. Binding of low affinity N-formyl peptide receptors to G protein. *J. Biol. Chem.* 270:1.
49. **Quehenberger, O., E. R. Prossnitz, S. L. Cavanagh, C. G. Cochrane, and R. D. Ye.** 1993. Multiple domains of the N-formyl peptide

receptor are required for high-affinity ligand binding. *J. Biol. Chem.* 268:18167.

50. **Quehenberger, O., E. R. Prossnitz, C. G. Cochrane, and R. D. Ye.** 1992. Absence of G<sub>i</sub> proteins in the Sf9 insect cells. *J. Biol. Chem.* 267:19757.

51. **Samama, P., S. Cotecchia, T. Costa, and R. J. Lefkowitz.** 1993. A mutation-induced activated state of the  $\beta$ 2-adrenergic receptor. *J. Biol. Chem.* 268:4625.

52. **Sanford, K. K., W. R. Earle, and G. D. Likely.** 1948. The growth in vitro of single isolated tissue cells. *J. Natl Cancer Inst.* 9:229.

53. **Saverese, T. M. and C. M. Fraser.** 1992. In vitro mutagenesis and the search for structure-function relationships among G protein-coupled receptors. *J. Biochem.* 283:1.

54. **Schiffmann, E., B. Corcoran, and S. M. Wahl.** 1975. N-formylmethionyl peptides as chemoattractants for leucocytes. *Proc. Natl. Acad. Sci. USA* 72:1059.

55. **Schreiber, R. E., E. R. Prossnitz, R. D. Ye, C. G. Cochrane, and G. M. Bokoch.** 1994. Domains of the human neutrophil N-formyl peptide receptor involved in G protein coupling. *J. Biol. Chem.* 269:326.

56. **Schreiber, R. E., E. R. Prossnitz, R. D. Ye, C. G. Cochrane, A. J. Jesaitis, and G. M. Bokoch.** 1993. Reconstitution of recombinant N-formyl chemotactic peptide receptor with G protein. *J. Leukoc. Biol.* 53:470.

57. **Scott, J. K. and G. P. Smith.** 1990. Searching for peptide ligands with an epitope library. *Science* 249:386.

58. **Showell, H. J., R. J. Freer, S. H. Zigmond, E. Schiffmann, S. Aswanikumar, B. Corcoran, and E. L. Becker.** 1976. The structure-activity relations of synthetic peptides as chemotactic factors and inducers of lysosomal secretion for neutrophils. *J. Exp. Med.* 143:1154.

59. **Sklar, L. A., A. J. Jesaitis, and R. G. Painter.** 1984. The neutrophil N-formyl peptide receptor: Dynamics of ligand-receptor interactions and

their relationship to cellular responses. In *Regulation of Leukocyte Function*. R. Snyderman, ed. Plenum Press, New York, p. 29.

60. **Snyderman, R. and M. C. Pike.** 1984. Transductional mechanisms of chemoattractant receptors on leukocytes. In *Regulation of leukocyte function*. R. Snyderman, ed. Plenum Press, New York, p. 1.

61. **Stoyanov, B., S. Volinia, T. Hanck, I. Rubio, M. Loubtchenkov, D. Malek, S. Stoyanova, B. Vanhaesebroeck, R. Dhand, B. Nürnberg, P. Gierschik, K. Seedorf, J. J. Hsuan, M. D. Waterfield, and R. Wetzker.** 1995. Cloning and characterization of a G protein-activated human phosphoinositide-3 kinase. *Science* 269:690.

62. **Uthayakumar, S. and B. L. Granger.** 1995. Cell surface accumulation of overexpressed hamster lysosomal membrane glycoproteins. *Cell. Mol. Biol. Res.* 41:405.

63. **Watson, J. D., M. Gilman, J. Witkowski, and M. Zoller.** 1992. In vitro mutagenesis. In *Recombinant DNA*. 2nd ed. Scientific American Books, New York, p. 191.

64. **Wheeler, G. L. and M. W. Bitensky.** 1977. A light-activated GTP-ase in vertebrate photoreceptors: regulation of light-activated cyclic GMP phosphodiesterase. *Proc. Natl. Acad. Sci. USA* 74:4238.

65. **Williams, L. T., R. Snyderman, M. C. Pike, and R. J. Lefkowitz.** 1977. Specific receptor sites for chemotactic peptides on human polymorphonuclear leukocytes. *Proc. Natl. Acad. Sci. USA* 74:1204.

# Production and Purification of Formyl...

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