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Cytoskeletal regulation of chemotactic receptors: Molecular complexation of N-formyl peptide receptors with G proteins and actin

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Abstract: Signal transduction via receptors for N-formylmethionyl peptide chemoattractants (FPR) on human neutrophils is a highly regulated process. It involves direct interaction of receptors with heterotrimeric G-proteins and may be under the control of cytoskeletal elements. Evidence exists suggesting that the cytoskeleton and/or the membrane skeleton determines the distribution of FPR in the plane of the plasma membrane, thus controlling FPR accessibility to different proteins in functionally distinct membrane domains. In desensitized cells, FPR are restricted to domains which are depleted of G proteins but enriched in cytoskeletal proteins such as actin and fodrin. Thus, the G protein signal transduction partners of FPR become inaccessible to the agonist-occupied receptor, preventing cell activation. We are investigating the molecular basis for the interaction of FPR with the membrane skeleton, and our results suggest that FPR, and possibly other receptors, may directly bind to cytoskeletal proteins such as actin.

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Introduction

Human neutrophils exploit the function of several receptor types to sense concentration gradients of chemoattractants, such as N-formylmethionyl peptides, complement fragment 5a (C5a), LTB_4, or platelet-activating factor (1). N-formylmethionyl peptide chemoattractant receptors (FPR) are among the most thoroughly studied members of the family of receptors coupled to guanyl nucleotide-binding proteins (G protein). Agonist binding to FPR and other chemoattractant receptors results in a variety of host defensive responses of neutrophils including chemotaxis, adhesion, superoxide production, and secretion of hydrolytic enzymes and microbicidal factors (2, 3).

The gene for FPR has recently been cloned (4) and sequenced. Its predicted amino acid sequence suggests that this receptor has seven transmembrane segments analogous to other receptors coupled to heterotrimeric G-proteins (5). Signal transduction by such receptors is highly regulated, allowing cellular adaptation of a variety of responses to a wide range of conditions. Desensitization is one such adaptive process that results in a blunted response in spite of the presence of agonist-occupied receptors. The mechanisms proposed to explain desensitization include receptor phosphorylation, sequestration, and down regulation (6, 7). The regulation of the level of G protein α subunits has also been implicated in desensitization (8). We have proposed a novel mechanism for desensitization of FPR in neutrophils which involves the submembranous cytoskeleton or membrane skeleton (MSK) of the neutrophil. The principle of this mechanism is that the physical segregation of different components of the signal transduction system into different plasma membrane domains controls their accessibility, and hence, interaction. This article summarizes our current understanding of this process.

Cytoskeleton and signal transduction

Abundant evidence exists suggesting interactions of a variety of receptors (9–13) and other components of signal transduction cascades (14–18) with the

MSK. Most of these reports describe such interactions, but the biochemical basis or functional consequences of the interactions are yet unclear. The MSK may serve as a general organizer of proteins in the plasma membrane and thus control protein interactions (19, 20). Abundant evidence also exists suggesting that receptor distribution on cell surfaces are laterally differentiated in the form of patches and caps both following and prior to receptor occupancy (21, 22).

Since Rodbell and coworkers recently proposed that G proteins may exist in polydisperse structures resembling microtubules (23–25), a new regulatory dimension has been added to transmembrane signaling as it defines G proteins as a cytoskeleton-like structure. This view implies a mechanism by which G proteins could be confined to certain plasma membrane domains. In neutrophils such mechanisms may be operative (26).

FPR signaling and the cytoskeleton

A number of experimental observations implicate the cytoskeleton in regulation of FPR signaling. The rate and duration of the neutrophil respiratory burst, which is one of the responses activated by the FPR system (27), is enhanced by dihydrocytochalasin B (dhCB), an alkaloid that disrupts microfilaments (28). Similar results were obtained after botulinum C2 toxin treatment of neutrophils, which results in ADP-ribosylation of actin (29) and its subsequent depolymerization. At physiological temperatures FPR becomes transiently associated with the cytoskeleton. This process is inhibited by cytochalasin B, thus suggesting involvement of the cytoskeleton in signal termination. At 15°C, a temperature permissible for desensitization but not internalization or secretion (30), FPR progressively associate with the cytoskeleton as they become desensitized (31). This association is also slowed by dhCB (30, 32) and inversely related to formyl peptide-stimulated superoxide production. Comparison of the number of receptors remaining uncomplexed with the cytoskeleton and the logarithm of rate of superoxide production reveals a linear relationship, suggesting that association of FPR with the cytoskeleton is indeed involved in desensitization of this receptor system (30).

Interaction of receptors with G proteins can modulate the two affinity states of receptors for their agonists. Agonists exhibit high-affinity binding when receptors are coupled to a G protein, while uncoupled receptors bind agonists with low affinity (33). GTP induces uncoupling of receptor and G protein and, therefore, shifts receptors to the low-affinity state for agonists. For FPR, a third affinity state has been observed which is characterized by a very low dissociation rate of bound ligand (31, 34). This "super high-affinity state" is detected when FPR become Triton-insoluble and hence, cytoskeleton-associated.

The molecular basis for the high affinity state is still unknown. Norgauer et al. (35) concluded that F-actin is not responsible for the regulation of the affinity states of FPR because botulinum C2 toxin did not influence binding characteristics of the receptor. However, botulinum C2 toxin treatment of neutrophils for 1 h could only destroy 75% of the F-actin, leaving the possibility that the remainder might be sufficient to regulate ligand binding to FPR. In addition, as discussed below, membrane skeletal actin has characteristics different from cytoplasmic F-actin (36). It demonstrates insensitivity to botulinum C2 toxin as well as cytochalasins (37) thus suggesting that all possibilities for a role for F actin have not yet been ruled out.

Plasma membrane domains and desensitization

A more refined characterization of the subcellular distribution of FPR led to discovery that there are plasma membrane fractions which can be distinguished by their different densities in isopycnic sucrose density gradient sedimentation studies (38). The lighter fraction (PML) contains the plasma membrane marker alkaline phosphatase and surface glycoproteins and is enriched in G proteins. In membranes obtained from neutrophils labeled with the FPR photoaffinity ligand, *f*MLFK-^{125}I-ASD (39), at 4°C, a temperature which prevents internalization of receptors and greatly slows desensitization, the receptor cosediments with PML on the sucrose gradients. However, photoaffinity labeling of neutrophils at 15°C, a temperature allowing desensitization to occur but not permissive for receptor internalization, the receptor distribution shifts to fractions of slightly higher density (PM-H). These membrane fractions are characterized by a significant enrichment of the cytoskeletal proteins actin and fodrin and are depleted in G-proteins (40). This result supports a role of the MSK in the desensitization process, providing for a physical mechanism for control, i.e. limitation of the accessibility of signal transduction partners by their lateral segregation or compartmentation in different membrane domains.

Analysis of the hydrodynamic behavior of FPR solubilized by octylglucoside (OG) from these two different membrane fractions provides additional support for this hypothesis. Soluble FPR exhibit distinct sedimentation behavior in detergent-containing sucrose gradients (38). The receptors from unstimulated cells which are found predominantly in the G protein-containing PML, sediment with an apparent

sedimentation coefficient of 7S while the receptors found in PM-H, along with cytoskeletal proteins, sediment like 4S particles. In membranes from desensitized neutrophils most of the receptors are found in PM-H and, accordingly, sediment at 4S. Uncoupling of FPR from G proteins upon desensitization has also been shown in HL-60 cells (41).

The 7S form of FPR can be shifted to the 4S form in the presence of GTP suggesting that the 7S form represents the G protein-coupled form of the receptor. This suggestion has now been confirmed by reconstitution of 7S form of FPR from the 4S form obtained from neutrophil membranes (42) or from membranes prepared from FPR transfected TX2 mouse fibroblast cells (43) using purified G protein from neutrophils and bovine brain. Furthermore, we have now shown that synthetic peptides mimicking portions of intracellular loops 2 and 3 and a region of the carboxyl terminal tail also disrupt 7S complexes of FPR and G (44, 45).

These results strongly support the hypothesis that the hydrodynamic forms found in the PM-H and PML represents the G protein uncoupled and coupled forms, respectively, of FPR. Thus, we hypothesize that FPR in responsive neutrophils can access G proteins in PML (46). Upon desensitization, the receptors are shifted to PM-H, by an unknown mechanism which most likely involves lateral diffusion and interaction of FPR with components of the MSK, thus restricting the interaction with G proteins (Figure 1). Recently a report by Johansson and co-workers confirmed the existence of regulated lateral diffusion of FPR, which at least qualitatively supports immobilization of FPR by the membrane skeleton under similar conditions (47).

This model can only be valid if G protein mobility in the membrane is restricted, preventing diffusion of FPR into the PM-H and if there is no intrinsic modification of the receptors from desensitized cells that prevents them from interacting with G protein. Indeed, some evidence exists indicating that G proteins are coupled to the cytoskeletal elements in an analogous manner as receptors. It has been shown that β subunits of G proteins co-fractionate with cytoskeletal actin upon differential detergent extraction (48). Several G_{α} subunits bind specifically to tubulin, suggesting a role for G protein-microtubule interaction in signal transduction (49, 50). In our own studies, we have found that a significant fraction of $G_{i\alpha}$ subunits are insoluble upon sedimentation of OG extracts of unstimulated membranes (Figure 2). Most recently, Sarndahl et al. (26) have confirmed such restricted mobility by showing that $G_{i\alpha 2}$ is association with the Triton X-100 insoluble cytoskeleton can be modulated by the stimulation state of the cell. Both the interaction of β subunits with actin and of α subunits with tubulin could provide important mechanisms to achieve lateral segregation of receptors and G proteins in different membrane domains.

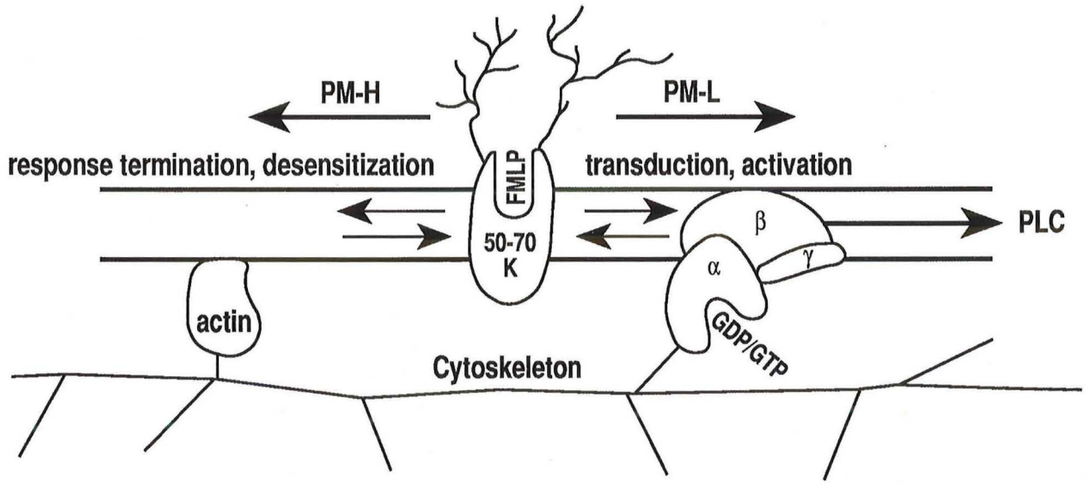

Fig. 1. Model of regulatory interactions of FPR with G proteins and actin. In responsive human, neutrophils most of the FPR are found in the light fraction of the plasma membrane (PM-L), which also contains most of the G proteins. A shift of FPR to the heavy plasma membrane fraction (PM-H), with a characteristic enrichment of cytoskeletal proteins, is observed as desensitization occurs. Rodbell's finding of polydisperse G protein structures provides an attractive basis for membrane compartmentalization with domains with G proteins allowing for signal transduction and domains with actin where receptors cannot access signal transduction partners. The polymeric structure of G proteins and actin would exclude mixing of these proteins by diffusion. The FPR, however, could diffuse between the different domains until agonist binding would permit interaction with G proteins or actin.

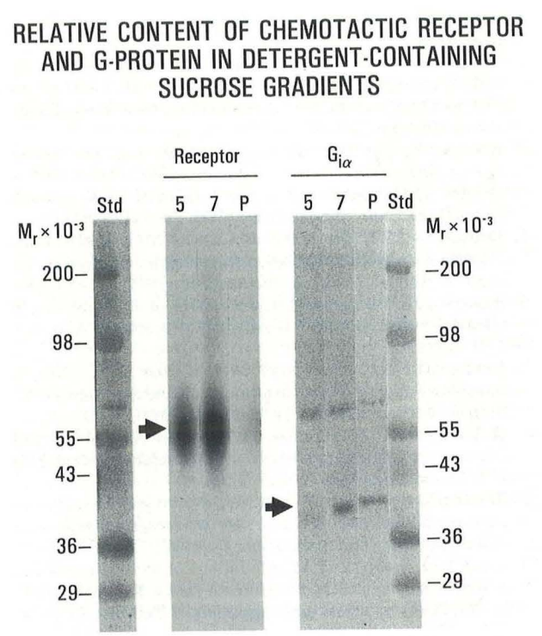

Fig. 2. Neutrophil G protein is associated with the membrane skeleton. The relative content of chemotactic receptor and G protein in linear sucrose gradients containing octyl glucoside prepared as reported previously (38) is shown. Approximately 50% of Giα is found to sediment in the pellet fraction (P) while the remainder cosediments with FPR as a soluble complex in fraction 7 (7S). Molecular weight standards flank the right and left group of three lanes from a nitrocellulose electrophoretic transfer used for western blot analysis (right) or autoradiography (left). The distribution of Giα as detected by antipeptide Giα antibodies and FPR as detected by autoradiography for photoaffinity-labeled FPR is shown as indicated.

FPR and the membrane skeleton

It appears to be reasonable to assume that the FPR in the PM-H fraction which is enriched in actin and fodrin represents a receptor form coupled to the MSK. Indeed, this concept is supported by several observations. The membrane skeleton is characterized by its insolubility in the detergent Triton X-100 (TX) by analogy to the cytoskeleton (50). Solubilization of plasma membranes from unstimulated human neutrophils in TX does not completely solubilize FPR. In contrast to experiments with OG, in which FPR are quantitatively solubilized, about 50% of the receptors are found in the pellet after sedimentation in sucrose gradients of membrane extracts in TX (51, 52). These pellets also contain a major portion of the membrane skeletal actin. Solubilization in the presence of agents which disrupt actin filaments, e.g. elevated concentrations of KCl, DNase I, or organic mercurial compounds result in parallel decreases in actin and FPR (> 50%) in the pellet fraction, suggesting that FPR are indeed linked to the membrane skeleton and that actin plays an important role for this linkage.

The functional significance for the observed coupling is supported by comparison of membranes from unstimulated neutrophils with membranes from desensitized cells. When neutrophils are photoaffinity labeled at 15°C, and thus desensitized, virtually all FPR are shifted to the membrane skeletal pellet (51, 52). This parallels the shift of FPR to PM-H in desensitized cells where the receptors have been found uncoupled from G protein (38). Interestingly, this receptor redistribution is insensitive to dhCB (see below). Cytochalasin-insensitive antigen-induced association of IgE with the membrane skeleton has also been described in RBL cells (13).

The molecular link of FPR to the membrane skeleton

The release of FPR from the membrane skeletal pellet with actin-disrupting agents suggests a critical role of actin for FPR "immobilization", although it does not exclude the possibility that other protein(s) may be involved. Recently, we have made several independent observations suggesting the FPR directly interacts with actin: first, FPR solubilized in OG from NaOH-treated membranes to remove endogenous actin and then transferred into TX, can interact with actin, as evidenced by an increase in the sedimentation rate of a significant fraction of FPR upon addition of exogenous purified neutrophil cytosolic actin; second, FPR solubilized from untreated membranes can be immunoprecipitated with anti-actin antibodies and the immune complexes of FPR-actin purified on protein A affinity matrices; third, in nitrocellulose overlays, photoaffinity-labeled FPR specifically binds to immobilized neutrophil actin; fourth, overlay binding can be inhibited by adding actin ($IC_{50} = 0.6\ \mu M$) to the liquid phase; and fifth, photoaffinity labeling of FPR in both actin-depleted, NaOH-treated membranes and detergent extracts thereof is increased by actin added back to the labeling cocktail with an EC_{50} of 0.1 μM, while other proteins such as ovalbumin, have no effect (53). Thus, despite the fact that actin is a "sticky" protein (54), the evidence is strong that actin can specifically bind to FPR in detergent solution (55) in a range that is not physiologically unreasonable.

These results support the hypothesis that actin may be the molecular link between the FPR and the bulk MSK. The actin effect on photoaffinity labeling, furthermore, suggests an actin-receptor interaction which may have a direct effect on receptor-ligand binding and thus appears to be of functional significance. The role of actin binding in the proposed model might be to remove the receptors from the G protein-containing domains and, thus, limit their access to signal transduction partners (Figure 1). The

model suggests that receptors may bind G protein or actin, opening speculation as to which receptor domains these regulatory proteins bind. An attractive possibility would be competition of G protein and actin for the same site on the receptor protein. Analysis of FPR sequence suggests that a 15 amino acid region (322–337) of the receptor carboxyl-terminal tail region that extends into the cytoplasm has significant (45–50%) identity to certain actin binding and cytoskeletal proteins such as vinculin (56), and coronin (57) as is shown below.

^{322}FPR^{336}	R A L T E D S T Q T S D T A T
^{433}VINCULIN^{447}	R S L G E I S A L T S K L A D
^{247}CORONIN^{262}	R A F T T P L S A Q V V D S A S

This region also participates in FPR interaction with G_{i2} and synthetic peptides mimicking this region specifically disrupt 7S FPR forms or inhibit the formation of reconstituted 7S FPR-G_i complexes (44). Sequence similarity studies between actin and $G_{i\alpha 2}$ also support this notion, as two decapeptide regions of $G_{i\alpha 2}$ and actin correspond very closely. The peptides ^{190}MKILTERGYS^{199} of actin and ^{53}MKIIHEDGYS^{62} of $G_{i\alpha 2}$ have 70% identity and 90% similarity. The actin stretch is located precisely adjacent to the actin-actin interaction site of actin polymers (58). The G_i stretch, though not yet identified as a functional interaction site of a G protein, is predicted to be adjacent to such a site in a recent three-dimensional model proposed by Deretic and Hamm (59).

Conclusion

Regulation of formyl peptide chemoattractant receptor may involve a novel mechanism that depends on FPR interaction with the membrane skeleton and a physical separation of FPR from G proteins. Such separation may be mediated by direct interaction of FPR with actin in the MSK. We speculate that actin and G protein may share sites on the FPR that permit G protein activation and immobilization to the MSK. Such sites would have to be functional as they would affect FPR interaction with its formyl peptide ligand.

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