# Neutrophil Chemoattractant Receptors and the Membrane Skeleton

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## Summary

**Signal transduction via receptors for N-formylmethionyl peptide chemoattractants (FPR) on human neutrophils is a highly regulated process which involves participation of cytoskeletal elements. Evidence exists suggesting that the cytoskeleton and/or the membrane skeleton controls the distribution of FPR in the plane of the plasma membrane, thus controlling the accessibility of FPR to different proteins in functionally distinct 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. The mechanism of interaction of FPR with the membrane skeleton is poorly understood but evidence is accumulating that suggests a direct binding of FPR (and other receptors) to cytoskeletal proteins such as actin.**


## Introduction

Neutrophil granulocytes or neutrophils are phagocytic cells which are an important component of the body's first line defense against invading microbes[1]. A number of highly specialized functions including chemotaxis, adhesion, superoxide production and secretion of hydrolytic enzymes allow these cells to accomplish this complicated task[2,3]. Neutrophils possess sensitive machinery that receives inflammatory signals which direct them to sites of infection. Microorganisms are then killed by release of hydrolytic enzymes, microbicidal peptides and toxic oxygen radicals (respiratory burst)[4]. Finally, the intruders are eliminated by phagocytosis. The potent bactericidal compounds produced by the neutrophil are non-selective agents and, therefore, their production has to be tightly regulated in order to be efficient and 'save' for the host organism.

Neutrophils express a number of different receptors for chemoattractants, which enable the cell to sense invading microbes and approach the site of infection by a directed migration[5]. This chemotactic process of guiding the cells along concentration gradients of chemoattractants is triggered by bacterial metabolites such as N-formylmethionyl peptides, which are derived from newly synthesized bacterial (or mitochondrial) proteins. In addition to bacterial mediators of chemotaxis, various inflammatory mediators, e.g. complement fragment 5a (C5a), leukotriene $B_4$ ($LTB_4$) or platelet-activating factor (PAF), are capable of activating neutrophils via specific receptors. Receptors for N-formylmethionyl peptides (FPR) are among the most thoroughly studied neutrophil receptors and are members of the family of receptors coupled to a guanyl nucleotide-binding protein (G protein). The FPR has recently been cloned[6] and exhibits seven hydrophobic domains, suggesting that this receptor has seven transmembrane segments, analogous to other G protein-coupled receptors[7,8].

Signal transduction via membrane-bound receptors is a highly regulated event which allows the cell to adapt its response to a wide range of conditions. Desensitization is such an adaptive process and results in a blunted response, in spite of the permanent presence of agonists. For G protein-coupled receptors, several mechanisms exist to accomplish the fine-tuning of the cellular response where either the access of ligand to receptor or the access of receptor to G protein is limited (for a review see refs 9 and 10). Receptor sequestration results in a decreased receptor number at the cell surface and, thus, in a reduced response. In another process, called down-regulation, receptors are degraded, resulting in an overall reduced number of cellular receptors. For some receptors like rhodopsin and the β-adrenergic receptor, phosphorylation increases the affinity of the receptor for regulatory molecules such as arrestin and arrestin-like proteins[11]. Binding of these phosphorylated receptors to arrestin-like proteins prevents interaction with a G protein and, therefore, interrupts the signaling cascade. The regulation of the level of G protein α subunits has also been implicated in desensitization[12].

In recent years we have proposed a new mechanism for desensitization of FPR in human neutrophils which involves the cytoskeleton and/or the membrane skeleton. The molecular events in this process may involve the segregation of different components of the signal transduction system into different plasma membrane domains. This article gives an overview of our current understanding of this new regulatory pathway, and suggests a possible general mechanism for modulation of chemoattractant receptor function.

## PMN, cytoskeleton and signal transduction

The stimulation of neutrophils is associated with the activation of motile functions and with dramatic morphological changes which implicate the cytoskeleton[14]. Shape changes are required for chemotactic mobility and phagocytosis, and secretion of vesicular contents involves transport of intracellular vesicles to the cell surface. At a subcellular level, one can observe subtle actin polymerization responses upon stimulation of neutrophils with chemoattractants[15]. An intriguing speculation is, therefore, that the cytoskeleton or the membrane skeleton participates in the regulation of receptors that are in turn sensors for the transfer of signals to the motile apparatus of the neutrophil.

Several lines of evidence indeed suggest that cytoskeletal elements play a role in receptor-mediated regulation of neutrophil function. Disruption of microfilaments with dihydrocytochalasin B (dhCB) or botulinum C2 toxin not only

directly affects shape and motility of the cells[16,17], but also affects receptor-mediated events like the respiratory burst[17-19]. Treatment of neutrophils with dhCB (or botulinum C2 toxin) increases the rate and duration of the respiratory burst, suggesting a role of microfilaments in desensitization.

The mechanism and site of action of these dhCB effects are unclear. One possibility would be that disruption of actin filaments prevents their functional interaction with FPR. Is there any evidence for an interaction of FPR with cytoskeletal elements which could provide the basis for a regulatory function? A number of reports from this and other laboratories have demonstrated an interaction between neutrophil FPR and cytoskeletal elements[20-22]. The association of FPR with the cytoskeleton parallels the desensitization of neutrophils and is inhibited by dHCB. Comparison of the number of receptors not complexed with the cytoskeleton and the rate of superoxide production reveals a quantitative relationship between these parameters, suggesting that association of FPR to the cytoskeleton is indeed implicated in desensitization of this receptor system[21]. There also exists abundant evidence for interactions of a variety of other receptors with the cytoskeleton or membrane skeleton[23-27]. Most of these reports only identify such interactions, and the molecular basis or functional consequences of the interactions are as yet unclear.

The cytoskeleton may also serve as a general organizer of proteins in the plasma membrane and allow or prevent certain proteins to interact[28,29]. It is conceivable, therefore, that the lateral interaction of receptors and G proteins is controlled by the cytoskeleton (or membrane skeleton). Ample evidence exists to suggest that receptor distribution on cell surfaces is laterally organized in the form of patches and caps, both following and prior to receptor occupancy[30-32]. The concept of lateral organization of a signaling cascade also requires that G proteins be restricted in mobility within the plane of the plasma membrane. In support of this concept, interactions of G proteins and other components of second messenger systems with cytoskeletal elements have been identified (see below)[33-38].

Since Rodbell and coworkers recently proposed that G proteins may exist in polydisperse structures resembling microtubules[39,40], a new dimension has been added to transmembrane signaling as G proteins are defined as cytoskeleton-like structures. This view would imply a mechanism by which G proteins could be confined to certain plasma membrane domains. In neutrophils such mechanisms appear to be operative.

## Plasma membrane domains and desensitization

It became clear that the fluid mosaic model of biological membranes, first introduced by Singer and Nicolson in 1972[41], had to be modified to accommodate the finding of proteins that could not freely diffuse within the plane of the membrane. It was well established, in the meantime, that functional domains exist in membranes which are caused by spatial restrictions for certain proteins. One mechanism enabling these restrictions is the interaction of membrane proteins with the cytoskeleton[42]. With information about the participation of cytoskeletal elements in the regulation of neutrophil function at hand, it was hypothesized that the cytoskeleton might control the lateral mobility of proteins implicated in signal transduction.

The characterization of the subcellular distribution of FPR in neutrophils led to the discovery of plasma membrane domains which can be distinguished by their different densities in isopycnic sucrose density gradients[43]. The fraction with the higher density (PM-H) is characterized by a significant enrichment of the cytoskeletal proteins actin and fodrin, whereas the lighter fraction (PM-L) is enriched in G proteins[43]. In responsive neutrophils, the majority of FPR is found in the PM-L fraction, along with the G proteins. However, in desensitized neutrophils, FPR are shifted to the PM-H fraction, suggesting that linkage of the receptor protein to the cytoskeleton provides a physical mechanism for lateral segregation of FPR and G proteins into different plasma membrane domains. Different physical interaction of FPR with G proteins was confirmed by sedimentation studies with FPR solubilized from the two membrane fractions[44]. The receptors solubilized from the predominantly G protein-containing PM-L fraction sediment with an apparent sedimentation coefficient of 7 S, while receptors found in the PM-H fraction, along with cytoskeletal proteins, sediment like 4 S particles. The 7 S form is shifted to the 4 S form in the presence of GTP, which is known to dissociate receptor-G protein complexes. This suggests that the 4 S form represents G protein-free receptors while the 7 S form represents the G protein-coupled form of the receptor. This suggestion has recently been confirmed by reconstitution of 7 S receptors from the 4 S form with purified G protein from neutrophil membranes ($G_n$) and purified $G_i$ from bovine brain[45].

These observations further support the hypothesis that physical segregation of FPR and G proteins into different membrane domains serves as a mechanism for desensitization and possibly response-termination[43,44,46]. In responsive neutrophils, most FPR are found in the PM-L fraction, where they can access G proteins. Upon desensitization, the receptors are shifted to the PM-H fraction, by an unknown mechanism, but which most likely involves lateral diffusion and interaction of FPR with components of the cytoskeleton or membrane skeleton, thus restricting the interaction with G proteins (Fig. 1). Recently, a report by Magnussen and coworkers confirmed that there is control of lateral diffusion of FPR, which at least qualitatively supports immobilization of FPR by the membrane skeleton under similar conditions[32].

This model can only be valid if a yet-to-be-discovered mechanism prevents G proteins from diffusing into the PM-H fraction. Indeed, some evidence exists indicating that G proteins are coupled to cytoskeletal elements, in an analogous manner to receptors. It has been shown that β subunits of G proteins co-fractionate with cytoskeletal actin upon differential detergent extraction[34]. Several $G_\alpha$ subunits bind specifically to tubulin, suggesting a role for G protein-microtubule interaction in signal transduction[36,37]. In our own studies we have found that a significant fraction of $G_i$ subunits are insoluble upon sedimentation of detergent extracts of unstimulated membranes. Both the interaction of β sub-

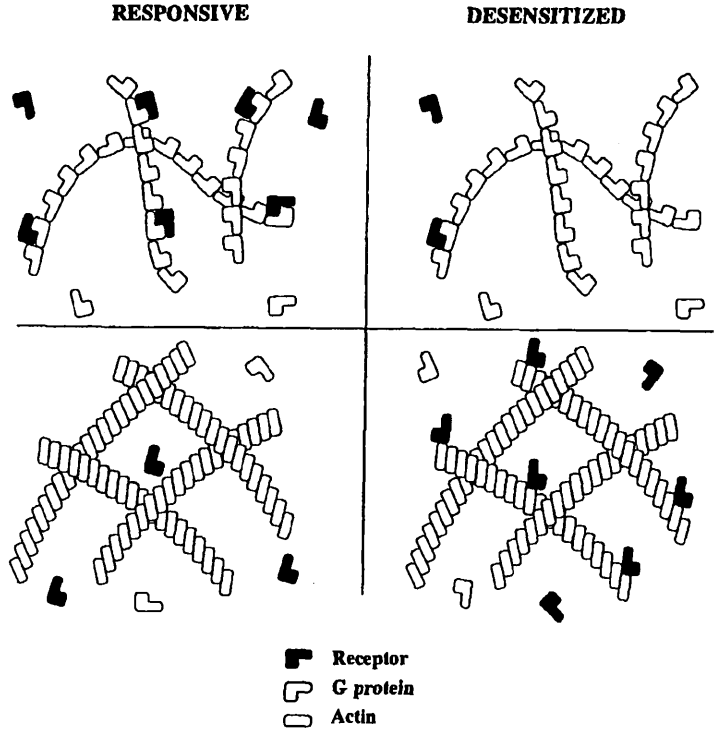


**Fig. 1.** Model of regulatory interactions of FPR with G proteins and actin. In responsive human neutrophils (left), most of the FPR are found in the light fraction of the plasma membrane (PM-L) which also contains most of the G proteins. A shift of FPR to the heavy plasma membrane fraction (PM-H) with a characteristic enrichment of cytoskeletal proteins, is observed as desensitization occurs. Rodbell's finding of polydisperse G protein structure[39,40] provides an attractive basis for membrane compartmentalization, with domains with G proteins allowing for signal transduction ('G domain') and domains with actin where receptors cannot access signal transduction partners ('A domain'). 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.

units with actin and of α subunits with tubulin could provide important mechanisms for achieving lateral segregation of receptors and G proteins in different membrane domains.

Another very intriguing mechanism for a defined distribution of G proteins in membrane compartments might be provided by the above-mentioned polydisperse form of G proteins[39,40,47]. The polymer structure of G proteins, which resembles actin and tubulin polymers, would prevent their diffusion and could serve to confine them to PM-L fractions. Thus, the cytoskeleton and cytoskeleton-like structures of G proteins would be crucial in accomplishing functional uncoupling of the receptors from their signaling partners (Fig. 1).

## FPR and the membrane skeleton

The hypothesis outlined above suggests that the regulatory events resulting in desensitization of FPR take place in the plasma membrane. It has been recognized in recent years that many cells possess a membrane skeleton which is a two-dimensional submembraneous actin network, distinct from the three-dimensional cytoskeletal network of microfilaments in the cytoplasm[48-50]. This structure has been implicated in the regulation of many cellular functions[50]. 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 membrane skeleton. Indeed, this concept is supported by several observations.

The membrane skeleton is characterized by its insolubility in the detergent Triton X-100 (TX), analogous to the cytoskeleton[49]. Solublization of plasma membranes from unstimulated human neutrophils in TX does not completely solubilize FPR. In contrast to experiments with octylglucoside, in which FPR are quantitatively solublized, about 50% of the receptors are found in the pellet after sedimentation in sucrose gradients of membrane extracts in TX[51]. 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, results in release of receptors from the pellet, suggesting that FPR are indeed linked to the membrane skeleton and actin plays an important role for this linkage (Table 1).

The functional significance of the observed coupling is supported by comparison of membranes from unstimulated neutrophils with membranes from desensitized cells. When neutrophils are desensitized, virtually all FPR are shifted to the membrane skeletal pellet (Table 1)[51]. This parallels the shift of FPR to the PM-H fraction in desensitized cells where the receptors have been found uncoupled from a G protein[44]. It is interesting to note that this receptor redistribution is insensitive to dhCB (see below). For IgE receptors an antigen-induced association with the membrane skeleton has been described, and this was also almost unaffected by various cytochalasins[26].

## 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 receptor 'immobilization', although it does not exclude the

**Table 1.** *Solubilization of neutrophil membranes*

| State of neutrophil | Solubilization condition | FPR in pellet (% of total) |
|---|---|---|
| Unstimulated | 0.5% TX | 50.3 ± 3.5 |
| Partially desensitized | 0.5% TX | 70.3 ± 3.1 |
| Fully desensitized | 0.5% TX | 93.2 ± 1.4 |
| Unstimulated | 0.5% TX/600 mM KCl | 21.8 ± 6.4 |
| Unstimulated | 0.5% TX/1 mM *p*CMPS | 21.7 ± 2.8 |
| Unstimulated | 0.5% TX/DNase I | 23.2 ± 0.8 |

Membranes were prepared from unstimulated, partially and fully desensitized neutrophils (for experimental details see ref. 51). The membranes were solubilized in 0.5% TX in the presence or absence of various actin-depolymerizing agents (KCl, DNase I and *p*-chloromercuriphenylsulfonic acid, *p*CMPS). The extracts were then spun in detergent-containing 5%-20% sucrose density gradients. The receptor distribution in the gradients was determined by laser densitometric scans of autoradigrams of SDS-PAGE gels of the fractions of the gradients. Values are means ± S.E.M. of 3-8 experiments.

possibility that other protein(s) are involved. Despite the fact that cellular actin concentrations are very high (several hundred μM) and actin is a 'sticky' protein, there is evidence that actin can specifically bind to FPR[52]. First, FPR solubilized from NaOH-treated membranes to remove endogenous actin can interact with exogenously added actin, as has been shown by an increased sedimentation rate of part of the receptors in the presence of actin. Second, FPR solubilized from untreated membranes can be immunosedimented with anti-actin antibodies. Third, in a nitrocellulose overlay assay, photoaffinity-labeled FPR specifically bind to neutrophil actin. This binding is inhibited by added actin, with an $IC_{50}$ of about 0.1 μM. Fourth, labeling of FPR with an agonistic photoaffinity ligand in both actin-depleted (NaOH-treated) membranes and detergent extract thereof is increased by actin added back to the incubation mixture, with an $EC_{50}$ of 0.1 μM, while addition of other proteins, e.g. ovalbumin, has no effect[52].

These results support the hypothesis that the FPR may be an actin-binding protein. In particular, the actin effect on photoaffinity labeling points to an actin-receptor interaction which 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 (Fig. 1).

This model suggests that receptors may bind alternatively to G protein or actin and opens speculation as to which receptor domains bind to these regulatory proteins. Our knowledge about interaction of receptors with G proteins has been greatly advanced in the last number of years[7,53,54], but no data are available on the molecular basis of receptor-actin interaction, which has only recently emerged as being of regulatory significance. An attractive possibility would be competition of G protein and actin for the same site on the receptor protein. Sequence similarity studies between actin and $G_i$ support this notion as two decapeptide regions of $G_i$ and actin correspond very closely. The peptides $^{190}$MKILTERGYS$^{199}$ of actin and $^{53}$MKIIHEDGYS$^{62}$ of $G_i$ have 70% identity and 90% similarity. The actin stretch is located precisely adjacent to the actin-actin interaction site af actin polymers. 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 Dereteic and Hamm[55]. Currently, we are attempting to explore the importance of this region using a synthetic peptide approach[13].

## Cytoskeleton or membrane skeleton?

The initial experiments pointing to a participation of cytoskeletal structures in FPR regulation were whole-cell studies[20-22] and the TX-insoluble pellet contained, therefore, the cytoskeleton as well as the membrane skeleton. A significant difference between the whole-cell approach and the studies with isolated plasma membranes is the dhCB sensitivity of the cytoskeletal association of FPR and the dhCB insensitivity of the membrane skeletal association. Although internalization is not an absolute prerequisite for desensitization, it might serve as a parallel or a downstream mechanism and thus explain the dhCB effect in whole cells. There is evidence that membrane skeletal actin in neutrophils is more stable than actin in the lamellipodia[16], which would explain the lack of an effect of dhCB on the membrane skeletal association of FPR. Various actin-binding and -crosslinking proteins that are present in the plasma membrane but not in the cytosol may contribute to different stabilities of membrane skeletal versus cytoskeletal actin[56]. Another more speculative explanation for different stabilities of different actin pools in the cell might be that they comprise different actin isoforms[57].

It is not clear at this point what role the three-dimensional cytosolic actin network plays in the regulation of FPR function. Current data support the concept of membrane skeletal actin being of crucial importance for FPR regulation. We believe the primary event in FPR desensitization to be the interaction of FPR with membrane skeletal actin, that results in immobilization of the receptors in a G protein-free membrane domain, thus interrupting the G protein-mediated signal transduction pathway.

## Receptor class desensitization

Two types of desensitization for G protein-coupled receptors have been characterized so far. Homologous desensitization only affects the receptor system which has been activated by an agonist, while heterologous desensitization also inactivates other receptors coupled to the same effector system[9,10]. Recently, a new type of desensitization was observed for chemoattractant receptors in neutrophils, resulting in desensitization of FPR and C5a receptors with either N-formylpeptides or C5a[58]. However, other receptors coupled to the same effector enzyme, phospholipase C in this case, were not affected. Although there is no experimental evidence so far, it is tempting to speculate that the observed coupling of FPR to the membrane skeleton might be the molecular basis for this newly discovered desensitization mechanism. A similar coupling for other chemoattractant receptors to the membrane skeleton remains to be demonstrated.

## Conclusion

The regulation of functional responses of human neutrophils

to N-formylmethinonyl peptides involves changed responsiveness of FPR to agonist occupancy. The desensitization of this receptor system may be accomplished by lateral segregation of components of the signaling cascade into different membrane domains. Actin in the actin-rich fraction of plasma membranes (PM-H), which is depleted of G proteins, appears to bind receptors after stimulation and, thus, prevents interaction with G proteins. The diffusion of the G proteins into the PM-H domain may be precluded by mechanisms involving binding to microtubules, or by polymerization to polydisperse structures which would be restricted to the PM-L domain. In a responsive cell, FPR have access to this domain and can activate G proteins. Although the detailed mechanism of the interaction of FPR with the membrane skeleton remains to be elucidated, a direct interaction of the receptor with actin emerges as a crucial link in this regulatory interaction.

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