Direct or C5a-induced Activation of Heterotrimeric G\textsubscript{i2} Proteins in Human Neutrophils Is Associated with Interaction between Formyl Peptide Receptors and the Cytoskeleton*

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The binding of ligands to N-formyl peptide chemoattractant receptors in human neutrophils results in a rapid association of these receptors with a cytoskeletal fraction and a specific activation and release of G\textsubscript{i2} α-subunits from this fraction. In the present study we could show that pretreating neutrophils with GDP\textsubscript{BS} prevented the fMet-Leu-Phe-induced association of its receptor with a cytoskeletal fraction and also blocked the release of G\textsubscript{i2} α-subunits from the same cytoskeletal fraction. In contrast, direct activation of G\textsubscript{i2} proteins by addition of GTP\textsubscript{S} or ALF\textsubscript{a} not only caused a release of G\textsubscript{i2} α-subunits from the cytoskeleton but also an association of formyl peptide receptors with the cytoskeleton. The receptor for complement fragment 5a, which transduces its signaling through the same G\textsubscript{i2} protein, triggers both a release of G\textsubscript{i2} α-subunits from the cytoskeleton fraction and, of even greater interest, an association between formyl peptide receptors and the cytoskeleton. The close relationship between the activation and release of G\textsubscript{i2} α-subunits from the cytoskeleton and the association of formyl peptide receptors with the cytoskeleton might, however, not be a matter of protein-protein exchange, since the increased binding of formyl peptide receptors to the cytoskeleton occurs more rapidly than the release of G\textsubscript{i2} α-subunits from the cytoskeleton. The present findings suggest a possible mechanism for the initiation of formyl peptide receptor desensitization during neutrophil locomotion.

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∥ The abbreviations used are: C5a, complement fragment 5a; FPR, formyl peptide receptor; fMet-Leu-Phe, N-formyl-L-methionyl-L-leucyl-\textsubscript{L}-phenylalanine; G-protein, GTP-binding protein; G\textsubscript{i2} α-subunit, α-subunit of the G\textsubscript{i2} protein; G\textsubscript{o,α} α-subunit of the G\textsubscript{o} protein; G\textsubscript{z,α} α-subunit of the G\textsubscript{z} protein; GDP\textsubscript{BS}, guanosine-5’-O-(3-thiotriphosphate); GTP\textsubscript{S}, guanosine-5’-O-(3-thiotriphosphate); PVDF, polyvinylidene difluoride; TX-100, Triton X-100.

To be able to move in a chemotactic gradient, motile cells like the human neutrophil must respond to changes in the concentration of one or probably several stimuli. In part, this is achieved by an adaptive process that results in a blunted response despite the permanent presence of agonists. This process, which is also called desensitization, involves receptor down-regulation by internalization as well as more rapid mechanisms by which components of the signaling pathway are modified. In general, two types of desensitization have been characterized, and these are designated homologous and heterologous (Sibley and Lefkowitz, 1985). Homologous desensitization affects only the receptor system that has been activated by an agonist, whereas heterologous desensitization also inactivates other receptors coupled to the same effector system. Both types of desensitization result in the uncoupling of receptors from their effector enzymes. Several potentially interrelated mechanisms for how the receptor-G-protein interaction are altered when cells get desensitized have been demonstrated and hypothesized (Sibley et al., 1985; McLeish et al., 1989).

Considering the neutrophil FPR, it has previously been suggested that binding to the cytoskeleton is an important step in the desensitization of this receptor to chemoattractant peptides (Je saisit et al., 1984; Je saisit et al., 1986). In addition to the association of FPRs with the cytoskeleton, a lateral segregation of the FPR from its G-protein has also been observed (Je saisit et al., 1989; for review, see Je saisit, 1992). Such segregation could well be a consequence of the N-formyl-L-methionyl-L-
leucyl-l-phenylalanine (fMet-Leu-Phe)-induced release of the α-subunits of the G2 protein (G2 α-subunits) from the cytoskeleton (Särndahl et al., 1993) and would, as part of the desensitization process, serve to keep the FPR from transducing its signal to its corresponding G-protein. Although both the activation and cellular handling of G2 proteins and FPRs are most likely of importance in the desensitization process, it is still unclear whether they are in any way interrelated.

The present study was performed to reveal whether or not the activation and release of G2 α-subunits from the cytoskeleton and the association of FPRs with the cytoskeleton are interdependent processes. This was done to gain further knowledge about the desensitization of FPRs that is necessary for neutrophil locomotion.

MATERIALS AND METHODS
Chemicals—All reagents used were of an analytical grade. Dextran and Ficoll-Paque were from Pharmacia Biotech (Sollentuna, Sweden), fMet-Leu-Phe was obtained from Sigma, and GDPβS and Ficoll-Paque were from Pharmacia Biotech (Sollentuna, Sweden), eton (Sa¨rndahl et al., 1993) and would, as part of the desensitization process, serve to keep the FPR from transducing its signal to its corresponding G-protein. Although both the activation and cellular handling of G2 proteins and FPRs are most likely of importance in the desensitization process, it is still unclear whether they are in any way interrelated.

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FIG. 1. Immunoblot analysis of the association of FPRs and G2 α-subunits with the cytoskeleton in GDPβS-pretreated neutrophils. The GDPβS pretreatment was carried out as described previously (Särndahl et al., 1989). Immediately after permeabilization, the neutrophils were exposed to 1 mM GDPβS for 10 min at 4 °C. The samples were then transferred to a 37 °C water bath, incubated for an additional 10 min, and subsequently stimulated with 20 nM fMet-Leu-Phe for 30 s. The stimulation was stopped by putting the cells on ice and simultaneously adding ice-cold TX-100-containing medium. Neutrophils termed “unstimulated” were not treated with GDPβS or fMet-Leu-Phe. The 1st and 2nd lanes, respectively, show the cytoskeletal fractions of unstimulated and fMet-Leu-Phe-stimulated GDPβS-pretreated neutrophils; for comparison, the 3rd lane shows the cytoskeletal fraction of fMet-Leu-Phe-stimulated permeabilized cells not treated with GDPβS. The proteins were detected with a 1:3000 dilution of the anti-FPR antibody (A) and a 1:2500 dilution of the anti-G2 α-antibody (R16, 17) (B).

RESULTS
Effects of GDPβS on the Association of FPRs and G2 α-Subunits—Cytoskeletal preparations were solubilized in sample buffer and boiled for 5 min. Thereafter, the proteins of each sample (1 × 10⁶ cell equivalents per lane) were separated on a 7.5 or 10% SDS-polyacrylamide gel to detect FPRs and G2 α-subunits, respectively. The proteins were then blotted onto a PVDF membrane as described previously (Särndahl et al., 1993). The membranes were blocked with 5% (w/v) bovine serum albumin in phosphate-buffered saline, pH 7.3, overnight at 4 °C, and then with 0.5% (w/v) dried milk for 30 min at 37 °C. The immunoblotting was performed by exposing the PVDF membranes to the different primary antibodies, after which a peroxidase-conjugated secondary antibody was added and the immune reaction was detected as enhanced chemiluminescence. When using the anti-FPR antibody, control experiments were performed by adding the antibody (1:300 dilution) to the FPR C-terminus peptide against which the antibody had been raised (10 μg/ml) for 24 h at 4 °C. This mixture was then added to the PVDF membrane, and the immune reaction was detected as described above. Densitometric analysis was performed with an Ultrascan XL enhanced laser densitometer (LKB, Bromma, Sweden).

Preparation of the Cytoskeletal Fractions—Cytoskeletal fractions were prepared using Triton X-100 (TX-100) as described by Särndahl et al. (1989, 1993). The obtained cytoskeletal preparations were washed once, pelleted, and prepared for electrophoresis by resuspending the pellets in a sample buffer previously described (Särndahl et al., 1993).
Effects of GTPγS and AlF₄⁻ on the association of FPRs and G₁₂α-subunits with the cytoskeleton. Activation with GTPγS and AlF₄⁻ was carried out as described previously (Särmårdh et al., 1993). Neutrophils were exposed to AlF₄⁻ (10 μM AlCl₃ + 20 mM NaF) for 20 min at 37 °C, immediately after permeabilization, to 100 μM GTPγS for 10 min at 4 °C and then transferred to a water bath and incubated at 37 °C for an additional 10 min. The stimulation was stopped by putting the cells on ice and simultaneously adding ice-cold TX-100-containing medium. Unstimulated indicates cytoskeletal fractions of permeabilized or unpermeabilized neutrophils not treated with GTPγS or AlF₄⁻ (1st and 4th lanes). 2nd and 5th lanes show the cytoskeletal fractions of GTPγS- and AlF₄⁻-treated neutrophils, respectively. For comparison, 3rd and 6th lanes show the cytoskeletal fraction of unstimulated fMet-Leu-Phe-stimulated cells. The proteins were detected with a 1:300 dilution of the anti-FPR antibody (A) and a 1:2500 dilution of the anti-G₁₂α-antibody (R16,17) (B).

induced release of the 40-kDa G₁₂α-subunit from the cytoskeleton (Fig. 1B). Densitometer analysis of these blots revealed that 94 ± 1% (mean ± S.E., n = 4) of the 40-kDa protein band remained associated with the cytoskeletal fraction in GDPβS-preincubated neutrophils. Comparison of the cellular handling of FPRs and Gi₂ proteins when measuring the effects of GDPβS and GDPγS on the association of chemotactic peptide receptors to the cytoskeleton and the activation and release of Gi₂ proteins when using a radiolabeled ligand to detect Gi₂ proteins, we confirm the results gained by direct manipulation of the G₁₂ proteins with GTPγS and AlF₄⁻ (Fig. 2).

Effects of GDPγS and AlF₄⁻ on the Association of FPRs and G₁₂α-Subunits with the Cytoskeleton—The association of FPRs with the cytoskeleton was further examined by incubating the cells with GTPγS or AlF₄⁻ to activate the G-protein in a ligand-independent manner. As shown in Fig. 2A (2nd and 5th lanes), GTPγS and AlF₄⁻, respectively, induced an association between the receptor and the cytoskeleton, even in the absence of fMet-Leu-Phe. These effects were quite comparable with the association obtained when using fMet-Leu-Phe alone (Fig. 2A, 3rd and 6th lanes). Parallel immunoblot analysis also confirmed that both GTPγS and AlF₄⁻ induced a release of G₁₂α-subunits from the cytoskeletal fraction (Fig. 2B, 2nd and 5th lanes) that was quite similar to that observed upon stimulation with fMet-Leu-Phe alone (Fig. 2B, 3rd and 6th lanes, and Fig. 1B). Densitometer analysis revealed that only 22 ± 7 and 26 ± 6% (mean ± S.E., n = 6 and 5) of the 40-kDa protein band remained associated with the cytoskeletal fraction after GTPγS or AlF₄⁻ activation, respectively.

Effects of C5a on the Association of FPRs and G₁₂α-Subunits with the Cytoskeleton—The receptor for C5a, which is known to couple to G₁₂ proteins, caused a release of G₁₂α-subunits from the cytoskeleton (Fig. 3B, 2nd lane) similar to that caused by fMet-Leu-Phe (Fig. 3B, 3rd lane, and Fig. 1B). Densitometer analysis revealed that only 33 ± 5% (mean ± S.E., n = 4) of the 40-kDa protein band remained associated with the cytoskeletal fraction after C5a stimulation. Of even greater interest, C5a stimulation led to an association between FPRs and the cytoskeleton (Fig. 3A, 2nd lane). Since these findings were obtained with a natural ligand that is known to activate Gi₂ proteins, we confirm the results gained by direct manipulation of the Gi₂ proteins with GTPγS and AlF₄⁻ (Fig. 2).

Temporal Changes in the Association of FPRs and G₁₂α-Subunits with the Cytoskeleton—To examine the kinetics of the release of Gi₂α-subunits and the interaction of FPRs with the cytoskeleton, neutrophils were stimulated with fMet-Leu-Phe at 15 °C. At that temperature, the receptor is converted into a high affinity form and becomes associated with the cytoskeletal fraction but is not internalized (Jesaitis et al., 1984; Sklar et al., 1984); in contrast, internalization occurs rapidly at 37 °C (t₁⁄₂ 15–20 s; Janeczek et al., 1989). Only very few FPRs could be detected in the cytoskeletal fraction of unstimulated neutrophils (Fig. 4A, inset, 1st lane). The fMet-Leu-Phe-induced association of FPR with the cytoskeletal fraction occurred rapidly and then leveled off (Fig. 4A, and inset, 2nd through 4th lanes). Concurrently, the number of 40-kDa Gi₂α-subunits associated with the cytoskeletal fraction of unstimulated neutrophils (Fig. 4B, inset, 1st lane) decreased upon stimulation with fMet-Leu-Phe. Comparison of the cellular handling of FPRs and G₁₂α-subunits reveals that the fMet-Leu-Phe-induced association of FPRs with the cytoskeleton precedes the release of Gi₂α-subunits from the same cellular fraction (Fig. 4).

DISCUSSION

Using the immunoblot technique and antibodies directed against the FPR or the G₁₂α-subunit, we studied the interdependence of the binding of chemotactic peptide receptors to the cytoskeleton and the activation and release of Gi₂α-subunits from the cytoskeleton. Exposing neutrophils to GDPβS, a GDP analogue that keeps the G-protein in an inactivated state, prevented the chemotactic peptide-induced release of Gi₂α-subunits from the cytoskeleton and also inhibited the association of FPRs with the cytoskeleton. These findings suggest that binding of FPRs to the cytoskeleton is regulated by a G-protein, as previously proposed (Särmårdh et al., 1989). In the cited investigation, we used a radiolabeled ligand to detect FPRs when measuring the effects of GDPβS; hence, it is possible that the results actually reflect a reduced binding affinity between the ligand and its receptor. This alternative interpretation is based on the fact that both GDPβS and GTPγS cause a concentration-dependent reduction in the affinity of the FPR for its ligand (Koo et al., 1983; Posner et al., 1994). However, in the present study we employed an immunoblot technique to directly detect the receptor protein itself and also found that GDPβS and GTPγS affected the FPR cytoskeletal association in completely different ways. We are therefore convinced that
the major effect of GDPβS, in this context, is to inhibit the association of FPR with the cytoskeleton. The impaired association of FPR with the cytoskeleton obtained by incubating the cells with GDPβS was obtained in the presence of the commonly used ligand fMet-Leu-Phe. On the other hand, and perhaps of greater interest in the present study, both GTPγS and AlF₄⁻ were found to induce association of FPRs with the cytoskeleton, even in the absence of any natural FPR ligand (Fig. 2A). This means that association of the FPR with the cytoskeleton requires neither the binding of a natural ligand nor the immediate conformational change that occurs in the receptor as a result of that protein-protein interaction. Furthermore, these findings are in agreement with a regulatory role of G-proteins in the process of FPR cytoskeleton association. The fact that stimulation with either GTPγS or AlF₄⁻ triggers an association between FPRs and the cytoskeleton and a parallel release of G₂α-subunits from the cytoskeleton (Fig. 2B) suggests that the FPR cytoskeletal association might be related to an activation-dependent release of G₂α-subunits from the cytoskeleton. Analysis of the time dependence of both the binding of FPRs to and the release of G₂α-subunits from the cytoskeleton (at 15 °C) revealed that the former association of FPRs occurred faster than the latter release of G₂α-subunits. This suggests that a mechanism more complex than a simple protein-for-protein exchange is involved in the FPR cytoskeletal association; however, such a conclusion might be uncertain due to the difference in number of FPRs and G₂α-subunits.

In this context, it should be mentioned that receptor phosphorylation is another step that is most likely involved in desensitization of the FPR, as has previously been demonstrated in the visual system (Wilden et al., 1986) and in the β-adrenergic receptor system (Lefkowitz et al., 1990). This supposition is based on the finding that the FPR in differentiated HL60 cells undergoes phosphorylation soon after stimulation (t₀ around 1 min, Tardif et al., 1993) and that there is a correlation between the phosphorylation and the desensitization of this receptor (Ali et al., 1993). Recently, it has also been suggested based on in vitro experiments that the FPR might be phosphorylated by a cytosolic kinase that has features similar to those of GRK2, a G-protein-coupled receptor kinase (Prossnitz et al., 1995). The present finding that G-protein activation could be an initial step in the association of FPRs with the cytoskeleton is compatible with the idea that phosphorylation of the FPR is a subsequent step in the desensitization process; this is true because an initial activation of G-proteins is known to activate several downstream protein kinases. A G-protein-induced phosphorylation of the FPR may be required to allow this receptor to become associated with the cytoskeleton and segregated from its G₂α protein (which at the same time is released from the cytoskeleton). If this is the case, then it is not surprising that desensitization had no effect on the affinity in FPR-G-protein interactions as seen in reconstitution experiments (Klotz and J. esaitis, 1994). Additional experiments are needed, however, to determine whether phosphorylation of FPR precedes or follows the association of FPR with the cytoskeleton.

Desensitization of the N-formyl peptide receptor was demonstrated several years ago in a number of laboratories (Showell et al., 1979; J. esaitis et al., 1986). Notwithstanding, the molecular basis of this process is still unknown, although both homologous and heterologous desensitization appear to be involved (Didsbury et al., 1991; McLeish et al., 1989). Interestingly, Didsbury and co-workers (1991) proposed that a third type of desensitization, referred to as “class desensitization,” also exists. These researchers observed that two chemotactic receptors in human neutrophils, namely the receptors for fMet-Leu-Phe and C5a, which are both coupled to the G₂α protein, are able to desensitize each other but that the α₁-adrenergic receptor, which is coupled to a different G-protein (i.e. G₃z), is not affected by pre-exposure to either fMet-Leu-Phe or C5a. Our experiments show that the FPR associates with the cytoskeleton not only when it is activated by its ligand but, more importantly, also when it is activated by another chemotactic stimulus, namely C5a. These findings are supported by the fact that the FPR also becomes associated with the cytoskeleton when the G₂α protein is directly activated by GTPγS or AlF₄⁻ in the absence of a ligand. Taken together, these
results agree with the idea of class desensitization (Didsbury et al., 1991) and suggest that the FPR can be desensitized by other receptors at the G-protein level. Moreover, in the present study stimulation with CSa released the α-subunits of its transducing G-protein (i.e. Gα2 protein) from the cytoskeleton. This finding provides additional support to the hypothesis that cellular segregation of FPRs and Gα2 proteins is an essential part of the mechanism underlying the termination and/or desensitization of FPR signaling properties (Jesaitis et al., 1989; for review see Jesaitis 1992). Furthermore, the previous observation that fMet-Leu-Phe-stimulation causes a selective release of Gα2 α-subunits without affecting the association of Gs α-subunits to the cytoskeleton (Särndahl et al., 1993) provides at least a partial explanation for the selectivity of class desensitization that is indicated in the model proposed by Didsbury and co-workers (1991).

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