Desensitization of N-Formylpeptide Receptor-mediated Activation Is Dependent upon Receptor Phosphorylation

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The human N-formylpeptide receptor (FPR) represents one of the most thoroughly studied leukocyte chemoattractant receptors. Despite this, little is known about the molecular mechanisms involved in the activation and desensitization of this receptor. To assess the role of phosphorylation in receptor function, U937 promonocytic cells were stably transfected to express the recombinant human FPR. Three mutant forms of the FPR lacking specific serine and threonine residues within the C terminus of the wild-type receptor. However, whereas the wild-type FPR was phosphorylated on both serine and threonine residues upon exposure to agonist and displayed a significantly reduced ability to stimulate G protein-mediated GTP hydrolysis, replacement of all 11 serine and threonine residues within the C terminus by alanine and glycine residues (∆ST) resulted in a receptor capable of ligand binding and G protein activation similar to the wild-type receptor. In addition to desensitization of G protein-mediated GTP hydrolysis, calcium mobilization was assayed to test whether desensitization occurred at a site distal to G protein activation. However, as observed with G protein activation, ∆ST underwent no desensitization of the calcium mobilization response upon a subsequent exposure to agonist. To define more precisely the role of specific serine and threonine residues within the C terminus of the wild-type receptor, two additional mutants were analyzed. Replacement either of Ser332, Thr334, Thr336, and Ser338 (mutant A) or of Thr334, Ser336, and Thr339 (mutant B) resulted in functional receptors that exhibited 50% of the level of phosphorylation following stimulation. Whereas mutant A, like ∆ST, could not be significantly desensitized by exposure to agonist, mutant B exhibited partial desensitization. These results indicate that phosphorylation of the FPR is a necessary and sufficient step in cellular desensitization, that multiple phosphorylation sites are involved, and that redundant desensitization does not occur downstream of G protein activation in the signaling cascade.

Neutrophils possess a large number of cell-surface G protein-coupled receptors that respond to structurally diverse ligands such as N-formylpeptides, complement components C5a and C3a, platelet-activating factor, and chemokines such as IL-8 and IL-8.

The abbreviations used are: IL-8, interleukin-8; FPR, N-formylpeptide receptor; GTPγS, guanosine 5′-O-(3-thiotriphosphate); fMLF, N-formyl-methionyl-leucyl-phenylalanine; Nle, norleucine; AMP-PNP, 5′-adenylyl β,γ-imidodiphosphate.

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Radioligand Binding—Ligand binding assays were performed on membranes prepared by nitrogen cavitation in a final volume of 0.2 ml of binding buffer. Membranes (30 μg of protein) were suspended in binding buffer (pH 7.4) consisting of 140 mM NaCl, 1.0 mM KH2PO4, 5 mM Na2HPO4, 1.5 mM CaCl2, 0.3 mM MgSO4, 1 mM MgCl2, and 0.2% bovine serum albumin. Binding buffer contained 10 mM sodium azide, 25 mM HEPES, 0.5% Nonidet P-40, 0.5 mM AMP-PNP, 0.25 mM ouabain, 1 mM MgCl2, 5 units/ml phosphatase, 5 units/ml phosphoramidate kinase, and 1 μM GTP (containing 2–4 μCi of [γ-32P]GTP). The incubation was carried out for 5–10 min at room temperature in the presence or absence of ligand. The assay was stopped with a 9-fold excess of ice-cold 5% (w/v) Norit A charcoal (J. T. Baker Inc.) in an aqueous solution containing 57 mM phosphoric acid. The charcoal and bound guanine nucleotides were sedimented by centrifugation, and the liberated [32P]phosphate in the supernatant was determined by scintillation counting.

Measurement of [Ca2+]i—Cells were harvested by centrifugation, washed once with phosphate-buffered saline, and resuspended at 5 × 106 cells/ml in Hanks’ buffered saline solution. The cells were incubated with 5 μM indo-1/AM for 25 min at 37°C, washed once with Hanks’ buffered saline solution, and resuspended at ~106 cells/ml in Hanks’ buffered saline solution containing 1.5 mM EGTA (pH 8.0). The elevation of intracellular Ca2+ by various amounts of fMLF was monitored by continuous fluorescent measurement using an SLM 8000 photon-counting spectrofluorometer (SLM-AMINCO) detecting at 400 and 490 nm, as described (18). The concentration of intracellular Ca2+ was calculated as described (22).

In Vivo Phosphorylation and Immunoprecipitation—FPR-transfected U937 cells were grown to a density of 1.0–1.5 × 106 cells/ml and washed three times with 150 mM NaCl and 10 mM HEPES (pH 7.4) to remove traces of phosphate. Cells were resuspended in phosphate-free RPMI 1640 medium containing 10 mM HEPES (pH 7.4) to a density of 106 cells/ml in a volume of ~0.5 ml to which was added 1 mM CaCl2 and 10 μCi/ml [32P]orthophosphate (23). Cells were stimulated with fMLF as indicated and immediately lysed by the addition of 0.33 volume of 4 × radioimmune precipitation assay buffer (40 mM Tris-HCl (pH 7.5), 600 mM NaCl, 4 mM EDTA, 0.4% SDS, 2% deoxycholate, 4% Triton X-100, 4 mM p-nitrophenyl phosphate, 40 mM sodium phosphate, 40 mM NaF, 20 μg/ml soybean trypsin inhibitor, 20 μg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, 400 ng/ml aprotinin, and 200 μg/ml pepstatin A). Following lysis and extraction for 10 min while rotating at 4°C, samples were centrifuged at 15,000 × g for 15 min at 4°C to remove insoluble debris. The supernatant was added to 10 mg of protein A-Sepharose that had been precoated with 15 μl of a rabbit antiserum directed against the C-terminal 12 amino acids of the FPR. The use of this antibody for immunoprecipitating the photoaffinity-labeled FPR has been previously described (23). Following binding for 1 h while rotating at 4°C, the beads were washed as follows: once with 1 ml of 50 mM Tris-HCl, 500 mM NaCl, 1% Triton X-100, and 0.2% SDS (pH 8.0); once with 1 ml of 50 mM Tris-HCl, 500 mM NaCl, 1% Triton X-100, and 0.1% SDS (pH 8.0); once with 1 ml of 50 mM Tris-HCl and 500 mM NaCl (pH 8.0); and finally with phosphate-buffered saline. Labeled peptide buffer (2 ml) was added to the concentrated samples and the samples were heated at 37°C for 10 min, followed by electrophoresis on a 12.5% SDS-polyacrylamide gel. Gels were dried, and relative determinations of [32P] content were performed with a Molecular Dynamics PhosphorImager.

Phosphoamino Acid Analysis—Phosphoamino acid content was determined as described (24). Briefly, following transfer of the SDS gel to
an Immobilon P membrane (Millipore Corp.), the immunoprecipitated band was excised and hydrolyzed in 100–200 μl of 6 N HCl for 1 h at 100–110 °C. The sample was dried in a SpeedVac concentrator; resuspended with 0.5 μg each of phosphoserine, phosphothreonine, and phosphotyrosine in a volume of 3–5 μl; and spotted onto a cellulose thin-layer plate (100 μm; EM Laboratories). Phosphoamino acids were separated by chromatography in 5:3 isobutyric acid/ammonium hydroxide (0.5 μl). Unlabeled phosphoamino acid standards were visualized with ninhydrin, whereas 32P-labeled phosphoamino acids were visualized with a PhosphorImager.

Desensitization—For desensitization of GTPase activity, cells were harvested, resuspended in RPMI 1640 medium containing 10 mM HEPES (pH 7.4) to a density of 10⁷ cells/ml, divided into two equal parts, and stimulated with either 1 μM fMLF or buffer for 10 min at 37 °C. Cells were then added to ice-cold buffer, harvested, and processed for membranes by nitrogen cavitation. Membranes from fMLF-stimulated and untreated cells were then assayed for GTPase activity as described above. For desensitization of the calcium mobilization response, cells (5 × 10⁶) were loaded with indo-1/AM as described above for calcium determinations and divided into two parts. One was stimulated with 1 μM fMLF for 10 min, whereas the other was treated with only buffer. The cells were then washed three times with Hanks’ buffered saline solution at room temperature to remove surface-bound fMLF and resuspended for assay of calcium mobilization as described above.

RESULTS

To examine the role of receptor phosphorylation in cellular desensitization in response to fMLF, a novel model system employing a human myeloid cell line was used. In addition to expressing the recombinant wild-type FPR, three mutant forms of the receptor were also expressed (Fig. 1). The first mutant, ΔST, had alanine or glycine substituted for each of the 11 serine and threonine residues of the carboxyl terminus. The high number of glycine residues used was due to the fact that 9 of 12 amino acids between Ser⁴²⁸ and Thr⁴³⁹ would have been alanine in the final receptor sequence. Given the helix-forming propensity of such an alanine-rich sequence, numerous serine and threonine residues were converted to glycine with the intent of eliminating any possible structural aberrations. In addition to ΔST, two mutants were generated changing only four Ser and Thr residues at a time (mutants A and B) (Fig. 1). These mutants, previously expressed as glutathione S-transferase fusion proteins containing the carboxyl-terminal 47 amino acids of the FPR, have been shown to represent potential sites of phosphorylation by GRK2 in vitro (13). Their role in desensitization was to be elucidated here.

Mutant forms of the FPR cDNA were generated by site-directed mutagenesis, subcloned into the mammalian expression vector pSFFV.neo, and introduced into the human myeloid cell line U937 by electroporation. Transfected cells were selected in the presence of G418 and analyzed for cell-surface expression of ligand binding by flow cytometry (Fig. 2). All three mutants demonstrated binding of the fluorescent ligand N-formyl-Nle-Leu-Phe-Nle-Tyr-Lys-fluorescein at levels comparable to those of the wild-type receptor. Expression of the wild-type FPR in U937 cells has previously been shown to yield binding affinities for [³H]fMLF similar to those of the FPR from dibutyryl cAMP-differentiated U937 cells and neutrophils (17). Detailed ligand binding studies performed here using [³H]fMLF demonstrated that the mutant forms of the FPR exhibited both high and low affinity binding sites similar to those of the wild-type FPR (Table I). In the presence of GTPγS, which irreversibly activates and dissociates G proteins, only one low affinity binding site was observed. These result are consistent with a fraction of the wild-type and mutant receptors being coupled to G proteins in the absence of GTPγS, suggesting that the mutant receptors might be capable of G protein activation.

To examine the functional coupling of the wild-type FPR as well as the mutant forms of the FPR, monitoring of intracellular calcium fluxes was performed with indo-1. Stimulation of each transfected cell line with 1 μM fMLF resulted in a rapid rise in intracellular calcium (Fig. 3). Stimulation of untransfected or vector-transfected cells yielded no such response, indicating that undifferentiated U937 cells do not express any FPR (data not shown). The rise in intracellular calcium was transient, returning to basal level within ~60 s. Taken together, these results indicate that the Ser and Thr residues of the carboxyl terminus of the FPR do not play a direct role in G protein activation or affect receptor function in such a way as to preclude ligand binding or signal transduction.

Previous data have suggested that the carboxyl terminus of the FPR is capable of being phosphorylated by a neutrophil kinase with properties similar to those of the G protein-coupled
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**TABLE I**

Ligand binding parameters of the wild-type and mutant forms of the FPR

| Receptor | Wild-type | ΔST | Mutant A | Mutant B |
|----------|-----------|-----|----------|----------|
| High affinity | Kₐ = 2.5 ± 1.2 nM | Kₐ = 1.8 ± 0.7 nM | Kₐ = 1.4 ± 0.5 nM | Kₐ = 1.7 ± 0.8 nM |
| B_max | 330 ± 147 fmol/mg | 548 ± 193 fmol/mg | 457 ± 156 fmol/mg | 301 ± 189 fmol/mg |
| Low affinity | Kₐ = 72 ± 38 nM | Kₐ = 46 ± 22 nM | Kₐ = 55 ± 24 nM | Kₐ = 62 ± 27 nM |
| +GTP·S | B_max = 1345 ± 580 fmol/mg | B_max = 1427 ± 711 fmol/mg | B_max = 1570 ± 503 fmol/mg | B_max = 1830 ± 791 fmol/mg |
| | B_max = 502 ± 247 fmol/mg | B_max = 875 ± 423 fmol/mg | B_max = 1412 ± 642 fmol/mg | B_max = 1773 ± 797 fmol/mg |

* High and low affinity binding were determined in the absence of GTP·S.

**FIG. 3.** Calcium mobilization of the wild-type and mutant forms of the FPR. For fMLF-stimulated elevation of intracellular calcium, cells expressing the wild-type (WT) and mutant forms of the FPR were loaded with indo-1/AM and stimulated with 100 nM fMLF at time = 20 s. Data are representative of four experiments. *mut A, mutant A; mut B, mutant B.*

**FIG. 4.** Phosphorylation of the wild-type FPR in transfected U937 cells. A, immunoprecipitation of the wild-type FPR from FPR- and vector-transfected U937 cells. Cells were loaded with [32P]orthophosphate and treated with either 1 μM fMLF (A, lanes 2–5; and B) or buffer (A, lane 1) prior to immunoprecipitation with antisera directed against the last 12 amino acids of the FPR as described under "Experimental Procedures." B, immunoprecipitation of the wild-type FPR from vector-transfected cells (lane 2) as compared with FPR-transfected cells (lane 1). C, phosphoamino acid analysis of the phosphorylated FPR. Following immunoprecipitation of the [32P]-phosphorylated FPR and separation on a 12% SDS-polyacrylamide gel, the proteins were transferred to an Immobilon P membrane, and the FPR protein band was excised and hydrolyzed as described under "Experimental Procedures." The circles indicate the positions of the unlabeled phosphoamino acid standards as determined with ninhydrin staining. PY, phosphotyrosine; PT, phosphothreonine; PS, phosphoserine.

receptor kinase GRK2 as well as by purified GRK2 itself (13).

To examine this reaction in vivo, transfected U937 cells were loaded with 32P-labeled inorganic phosphate, stimulated with fMLF, and extracted with detergent for immunoprecipitation of the FPR. Compared with unstimulated FPR-transfected U937 cells (Fig. 4A, lane 1), fMLF-stimulated cells showed a diffuse band with a molecular mass of 50–70 kDa (lane 2). This diffuse band has previously been shown to represent the glycosylated form of the FPR. Immunoprecipitation of the phosphorylated FPR was blocked by preincubation of the anti-FPR antiserum with the C-terminal peptide used as antigen (Fig. 4A, lane 3), but not by a peptide from the third intracellular loop (lane 4). The phosphorylated FPR was also not immunoprecipitated from FPR-transfected cells by preimmune serum (Fig. 4A, lane

5) or from vector-transfected U937 cells by the anti-FPR antiserum (Fig. 4B, lane 2), again confirming the absence of the FPR in the latter cells.

To determine the identity of the phosphorylated amino acids, acid hydrolysis was performed on the isolated phosphorylated FPR. Chromatographic separation of the phosphoamino acids revealed that both serine and threonine residues were phosphorylated, with no phosphotyrosine detected. Approximately equal amounts of the two phosphoamino acids were detected (55% phosphothreonine and 45% phosphoserine), similar to what was demonstrated for GRK2-mediated in vitro phosphorylation of the isolated FPR carboxyl terminus. Phosphorylation of the wild-type FPR in response to fMLF occurred in a dose-dependent manner (Fig. 5A). The EC₅₀ for phosphorylation was similar to the EC₅₀ for calcium mobilization, ~2 × 10⁻⁸ M, suggesting that the ligand-induced active G protein coupling state of the FPR also represented the substrate for the kinase. Phosphorylation was rapid, with a half-time of ~1–2 min (Fig. 5B).

Analysis of the site-directed mutants revealed that the ΔST mutant was not phosphorylated even at the highest concentra-
Recent results have suggested that desensitization of chemoattractant-mediated signaling can also occur at a site following the activation of G proteins and prior to the activation of phospholipase C. To test this possibility, cells expressing the wild-type and mutant receptors were treated with a saturating dose of fMLF (1 μM) for 10 min at 37 °C. This treatment is more than sufficient to achieve maximal phosphorylation of the wild-type receptor (see Fig. 5). Following this, cells were cooled to 0 °C, and membranes were prepared as described for ligand binding. Parallel (non-desensitized) controls were prepared that were not treated with fMLF. Determination of the ability of the wild-type FPR to stimulate G protein-mediated GTP hydrolysis in non-desensitized control membranes demonstrated that fMLF stimulation resulted in a 2-fold increase in GTP hydrolysis over the background of unstimulated membranes. This level of stimulation in the presence of fMLF was similar for non-desensitized cells expressing ΔST, mutant A, and mutant B (data not shown). However, when membranes from fMLF-treated (i.e., desensitized) cells expressing the wild-type FPR were assayed, the extent of GTP hydrolysis was reduced by ~80%, reflecting desensitization of the receptor-mediated activity (Fig. 7). In contrast, when membranes from fMLF-treated, desensitized cells expressing the ΔST mutant were compared with membranes from non-desensitized cells, only a slight reduction in the level of GTPase activity was observed (Fig. 7). This result suggests that phosphorylation of the carboxyl terminus of the FPR is required for desensitization of receptor-G protein coupling. Further analysis of mutants A and B revealed that both of these mutants were also deficient in their ability to undergo desensitization, although not to the same extent as the ΔST mutant, suggesting that phosphorylation within both sites A and B is necessary for maximal desensitization.

Recent results have suggested that desensitization of the Formylpeptide Receptor (FPR) occurs in a hierarchical manner, with residues within mutant A (site A) being phosphorylated before residues within mutant B (site B) (13).

To determine the possible role of phosphorylation in desensitization, alterations in the profile of ligand-induced G protein activation following desensitization were investigated. Desensitization was accomplished by treating cells with saturating doses of fMLF (1 μM) for 10 min at 37 °C. This treatment is more than sufficient to achieve maximal phosphorylation of the wild-type receptor (see Fig. 5). Following this, cells were cooled to 0 °C, and membranes were prepared as described for ligand binding. Parallel (non-desensitized) controls were prepared that were not treated with fMLF. Determination of the ability of the wild-type FPR to stimulate G protein-mediated GTP hydrolysis in non-desensitized control membranes demonstrated that fMLF stimulation resulted in a 2-fold increase in GTP hydrolysis over the background of unstimulated membranes. This level of stimulation in the presence of fMLF was similar for non-desensitized cells expressing ΔST, mutant A, and mutant B (data not shown). However, when membranes from fMLF-treated (i.e., desensitized) cells expressing the wild-type FPR were assayed, the extent of GTP hydrolysis was reduced by ~80%, reflecting desensitization of the receptor-mediated activity (Fig. 7). In contrast, when membranes from fMLF-treated, desensitized cells expressing the ΔST mutant were compared with membranes from non-desensitized cells, only a slight reduction in the level of GTPase activity was observed (Fig. 7). This result suggests that phosphorylation of the carboxyl terminus of the FPR is required for desensitization of receptor-G protein coupling. Further analysis of mutants A and B revealed that both of these mutants were also deficient in their ability to undergo desensitization, although not to the same extent as the ΔST mutant, suggesting that phosphorylation within both sites A and B is necessary for maximal desensitization.

Recent results have suggested that desensitization of the chemoattractant-mediated signaling can also occur at a site following the activation of G proteins and prior to the activation of phospholipase C. To test this possibility, cells expressing the wild-type and mutant receptors were treated with a saturating concentration of fMLF for 10 min (A) or with 1 μM fMLF for the indicated times (B). The FPR was immunoprecipitated and analyzed for its degree of phosphorylation following separation on a 12% SDS-polyacrylamide gel and PhosphorImager analysis.

Western blot analysis of fMLF-stimulated, G protein-mediated GTPase activity. Wild-type (WT) or mutant FPR-transfected cells were incubated with either buffer or 1 μM fMLF for 10 min at 37 °C to generate non-desensitized and desensitized cells, respectively. Membranes were then prepared and assayed for fMLF-stimulated GTP hydrolysis as described under "Experimental Procedures." Basal levels of GTP hydrolysis were identical in both fMLF-treated (i.e., desensitized) and untreated (i.e., non-desensitized) membranes. To determine the degree of desensitization, the amounts of fMLF-induced GTP hydrolysis were compared in the fMLF-treated (i.e., desensitized) and buffer-treated (i.e., non-desensitized) membranes. Desensitization (%) is defined as 100 × ([N − D]/N), where N represents the fMLF-stimulated GTP hydrolysis of buffer-treated (i.e., non-desensitized) membranes, and D represents the fMLF-stimulated GTP hydrolysis of fMLF-treated (i.e., desensitized) membranes.

Fig. 6. Phosphorylation of the wild-type and mutant forms of the FPR in transfected U937 cells. Transfected U937 cells were loaded with [32P]orthophosphate and treated with either 1 μM fMLF or buffer (for basal phosphorylation) prior to immunoprecipitation. Immunoprecipitated wild-type (WT) and mutant receptors were separated on 12% SDS-polyacrylamide gels and analyzed for incorporation of [32P]phosphate with a PhosphorImager.
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...identical to that of the wild-type receptor.

wild-type (U937 cells expressing the wild-type and mutant forms of the FPR.

fMLF being required to obtain a response to ligand, which resulted in a significantly higher concentration (30–50-fold) of calcium mobilization in response to the indicated doses of fMLF. Data are representative of four experiments with similar results.

**FIG. 8.** Desensitization of calcium mobilization by the wild-type and mutant forms of the FPR. U937 cells expressing the wild-type (WT) and mutant forms of the FPR were analyzed for fMLF-stimulated desensitization of calcium mobilization. Following loading with indo-1/AM, cells were stimulated either with (●) or without (○) 1 μM fMLF for 10 min at 37°C. Cells were then washed three times at room temperature to remove fMLF and subsequently assayed for calcium mobilization in response to the indicated doses of fMLF. Data are representative of four experiments with similar results. mut A, mutant A; mut B, mutant B.

dose of fMLF for 10 min at 37°C to desensitize them as described above, washed extensively to remove the ligand, and assayed for calcium mobilization, the result of phospholipase C activation. Desensitized cells were then compared with cells that had not been desensitized. When U937 cells transfected with the wild-type FPR were examined, desensitization resulted in a significantly higher concentration (30–50-fold) of fMLF being required to obtain a response to ligand, which at its maximum was less than half that obtained with the non-desensitized receptor (Fig. 8). When cells expressing the ΔST mutant were examined, no such change in the calcium mobilization response was observed. In fact, ΔST was completely refractory to desensitization, exhibiting an identical dose-response curve after the “desensitizing” treatment as compared with the untreated mutant cells, which themselves were indistinguishable from the untreated wild-type receptor. These results indicated that, whereas the Ser and Thr residues of the carboxyl terminus of the FPR play no role in the ability of the receptor to activate G protein (Figs. 3 and 8), they are essential to the process of desensitization, presumably through their ability to become phosphorylated. Analysis of mutant A also demonstrated no decrease in responsiveness following treatment with fMLF, indicating that mutation of the Ser and Thr residues within site A is sufficient to reproduce the effects seen with ΔST. Mutant B, however, yielded an intermediate profile between the wild-type receptor and the ΔST mutant. Although the maximal response following desensitization approached the level of the untreated cells, 5–10-fold higher concentrations of fMLF were required to achieve this level of response. The EC₅₀ values for untreated mutants A and B were essentially identical to that of the wild-type receptor.

**DISCUSSION**

In this report, the relationship between chemoattractant receptor phosphorylation and the desensitization of downstream signaling was investigated. Although it has long been known that stimulation of the FPR and other chemoattractant receptors results in desensitization of neutrophil functions, the mechanisms responsible for this desensitization are poorly understood (25, 26). Studies with membranes from fMLF- and C5a-desensitized leukocytes originally revealed that coupling between the FPR and G proteins was impaired under conditions of desensitization (27, 28). More recently, it has been demonstrated that desensitization of the FPR by C5a or IL-8 can occur in the absence of FPR phosphorylation, resulting in decreased inositol 1,4,5-trisphosphate generation and calcium mobilization without desensitization of G protein activation (16). Such results have led to the conclusion that desensitization of peptide chemoattractant receptors occurs downstream of G protein activation, possibly at the level of phospholipase C activation. In addition to this mechanism of desensitization, protein kinase C-mediated receptor phosphorylation, in the case of the C5a and IL-8 receptors, although not the FPR, appears to ameliorate receptor signaling. Since the FPR and the C5a and IL-8 receptors utilize similar if not identical signal transduction pathways, any downstream desensitization initiated by one of these receptors should be similarly initiated by the others. Furthermore, downstream desensitization must also be present under the conditions of homologous desensitization if the activating peptide chemoattractant receptors cannot be “distinguished between.”

The results presented in this paper demonstrate that the mechanisms of desensitization are not this simple. By completely preventing phosphorylation of the FPR with the ΔST mutant, not only was homologous desensitization of G protein activation abolished, but so was the downstream calcium mobilization response. This result demonstrates that activation by the FPR does not lead to the activation of a redundant downstream desensitization mechanism prior to the site of calcium mobilization (16). Two possible reasons exist to explain this result. First, it may be that the FPR does not activate the downstream desensitization machinery, as do the IL-8 and C5a receptors, or second, that it can circumvent the effects of this process under the conditions of homologous desensitization. If the former were true, then a model in which FPR signaling is different from IL-8 and C5a signaling would need to be invoked. To date, there is no evidence to substantiate this idea. If the latter were true, then a model in which heterologous desensitization can be distinguished from homologous desensitization would need to be created. In such a model, only when the second stimulus came from a receptor different from the first would the cell exhibit desensitization.

Further details of the mechanism of desensitization were revealed by the characteristics of the two partially phosphorylation-defective mutants, A and B. Mutants A and B both exhibited significant reductions (~50%) in the level of phosphorylation following fMLF stimulation. The discrepancy between the levels of phosphorylation of mutant A in vivo (~50%) and in vitro (~20%) may be a result of the influence of other regions of the FPR or of accessory proteins, such as arrestin-related proteins. Although phosphorylation of purified rhodopsin has been shown to proceed to a stoichiometry as high as 7–9 mol/mol receptor (29), arrestin was found to limit phosphorylation to 1–3 mol/mol rhodopsin (30, 31). Arrestin has also been found to promote the initial phosphorylation of rhodopsin (30). Thus, it is unclear exactly what effect on phosphorylation would be expected after substitution of Ser and Thr residues. In fact, if an arrestin homologue were to bind preferentially to the FPR when phosphorylated at site A as opposed to site B, it might be predicted that site B would become hyperphosphorylated in mutant A when compared with the wild-type FPR or mutant B.
The effects of the mutations on desensitization were clear, however. Mutant A as well as mutant B, to a slightly lesser extent, exhibited a decrease in the level of desensitization of G protein activation, suggesting that phosphorylation within both these sites is necessary for complete desensitization. However, when calcium mobilization was evaluated, mutant A demonstrated a complete lack of desensitization, identical to that seen with ΔST, whereas mutant B exhibited only a partial defect in the ability to undergo desensitization. These results indicate that a difference exists in the ability of the two sites to promote desensitization. Phosphorylation of site A appears to be critical in desensitizing the FPR since although mutant A demonstrated ~50% the level of phosphorylation as compared with the wild-type receptor, phosphorylation of site B resulted in no desensitization. Phosphorylation of only site A can result in partial desensitization of the FPR, as observed with mutant B. This desensitization was incomplete, however, indicating that for complete desensitization, residues within both sites A and B must be phosphorylated. These results are consistent with a sequential model of phosphorylation previously proposed, where residues within site A are phosphorylated, first resulting in partial desensitization with subsequent phosphorylation of residues within site B, leading to complete desensitization.

The results presented here demonstrate that the recombinant FPR expressed in U937 cells undergoes ligand-stimulated phosphorylation and desensitization as it does in the neutrophil. Furthermore, it was demonstrated that receptor phosphorylation is required for desensitization of immediate responses such as G protein activation as well as downstream responses such as calcium mobilization. The proposed model, in which chemoattractant-induced desensitization of calcium mobilization occurs in the absence of receptor phosphorylation, appears inconsistent with the results presented here. Further studies will be necessary to determine if perhaps homologous and heterologous desensitization mechanisms involve different pathways.

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