Differential Phosphorylation Paradigms Dictate Desensitization and Internalization of the N-Formyl Peptide Receptor*

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Following activation by ligand, most G protein-coupled receptors undergo rapid phosphorylation. This is accompanied by a drastic decrease in the efficacy of continued or repeated stimulation, due to receptor uncoupling from G protein and receptor internalization. Such processing steps have been shown to be absolutely dependent on receptor phosphorylation in the case of the N-formyl peptide receptor (FPR). In this study, we report results that indicate that the mechanisms responsible for desensitization and internalization are distinct. Using site-directed mutagenesis of the serine and threonine residues of the FPR carboxyl terminus, we have characterized regions that differentially regulate these two processes. Whereas substitution of all 11 Ser/Thr residues in the carboxyl terminus prevents both desensitization and internalization, substitution of four Ser/Thr residues between positions 328–332 blocks desensitization but has no effect on internalization. Similarly, substitution of four Ser/Thr residues between positions 334 and 339 results in a deficit in desensitization but a gain of internalization. These results indicate that phosphorylation at either site evokes receptor internalization, whereas maximal desensitization requires phosphorylation at both sites. These results also indicate that receptor internalization is not involved in the process of desensitization. Further analysis of the residues between 328–332 revealed that restoration either of Ser328 and Thr329 or of Thr331 and Ser332 was sufficient to restore desensitization, suggesting that phosphorylation within either of these two sites, in addition to sites between residues 334 and 339, is sufficient to produce desensitization. Taken together, these results indicate that the mechanisms involved in FPR processing (uncoupling from G proteins and internalization) are regulated differentially by phosphorylation at distinct sites within the carboxyl terminus of the FPR. The relevance of this paradigm to other G protein-coupled receptors is discussed.

The human N-formyl peptide chemoattractant receptor (FPR) is a member of the seven-transmembrane receptor superfamily. Expressed predominantly on leukocytes, the FPR, which binds ligands such as N-formyl-Met-Leu-Phe (fMLF), couples to heterotrimeric G proteins, activating numerous effectors including phospholipase C and thereby initiating responses such as chemotaxis, superoxide production, and degranulation (1). The FPR is one of the better studied of the chemoattractant/chemokine family of receptors, which is responsible, in large part, for control of numerous immune functions (2). Much interest has recently been devoted to this receptor family following the discovery that a number of chemokine receptors are co-receptors for human immunodeficiency virus (3). Despite the importance of this family of receptors, relatively little is known regarding the molecular mechanisms involved in activating and terminating receptor function.

Control and termination of effector functions, through a process termed adaptation, is essential to cellular function and occurs in the continued presence of activating ligand to prevent damage to the host. The mechanisms responsible for these processes, although poorly understood, are believed to consist of two essential components: uncoupling of the receptor from G proteins (desensitization) and removal of the receptor from the cell surface through receptor internalization (4, 5). Phosphorylation of the carboxyl terminus of the FPR is proposed to play a major role in these processes for this receptor (6). Members of the family of G protein-coupled receptor kinases have been shown to phosphorylate serine and threonine residues in the carboxyl terminus of the FPR (6). Substitution of all 11 serine and threonine residues in the carboxyl terminus with alanine and glycine residues produces a receptor incapable of undergoing either functional desensitization (uncoupling from G proteins) or ligand-induced internalization (7, 8). We have recently shown, however, that receptor phosphorylation, desensitization, and internalization are not obligatory steps for chemotaxis (8).

Studies with glutathione S-transferase fusion proteins containing the 47 amino acid carboxyl terminus of the FPR suggested that phosphorylation of the FPR occurs in a hierarchical manner within a region in which 8 out of 12 continuous residues (between residues 328–339) are either serine or threonine (6). The presence of acidic amino acids at the beginning and in the middle of this region is consistent with the demonstrated preferential recognition sites for phosphorylation by G protein-coupled receptor kinase 2 (9), suggesting that either or both of the clusters of Ser and Thr residues following an acidic residue may be sites of phosphorylation. Our experimental results indicated that significant phosphorylation occurred within each of these clusters of serine and threonine residues (7). However, the functional consequences of phosphorylation at specific sites within the carboxyl terminus are not known.

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1 The abbreviations used are: FPR, N-formyl peptide receptor; fMLF, N-formyl-methionyl-leucyl-phenylalanine; G protein, guanine nucleotide-binding regulatory protein; HBSS, Hanks’ balanced salt solution; m2AChR, m2 muscarinic acetylcholine receptor; GPCR, G protein-coupled receptor; Nleu, norleucinyl.
In this study, we have examined a series of FPR mutants with substitutions of carboxyl-terminal serine and threonine residues and determined the role(s) of these residues in receptor desensitization and internalization. The results of our study indicate that FPR desensitization and internalization must occur via distinct mechanisms due to the particular properties of individual phosphorylation-deficient mutants. Furthermore, our results demonstrate that receptor internalization is not required for desensitization to occur, further supporting the idea that these two processes occur via distinct mechanisms.

**EXPERIMENTAL PROCEDURES**

**Materials—**fMLF and fetal bovine serum were purchased from Sigma. N-Formyl-Neu-Leu-Phε-Neu-Tyr-Lys-fluorescein and indomethacin were obtained from Molecular Probes, Inc. (Eugene, OR). RPMI 1640 was from HyClone.

**Construction and Expression of Site-directed Mutants in U937 Cells—**The FPR gene was mutagenized as described previously (10). For transfection into U937 cells, 4 × 10⁶ cells grown in RPMI 1640 (supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 10 μg/ml streptomycin, 100 μg/ml HEPES, pH 7.4, and 10% fetal bovine serum) were centrifuged and resuspended with 400 μl of RPMI 1640 containing 10 mM glucose and 0.1 mM diethylenetriamine. Linearized DNA (5–20 μg) was added, and the cells were electroporated with a 240-V pulse from a 960-microfarad capacitor (resulting in a pulse time constant of 30–35 ms) and returned to 5 ml of growth medium (11). For selection, G418 was added to a final concentration of 1 mg/ml. Cells were cultured at 37 °C in a humidified atmosphere of 6% CO₂.

**Flow Cytometry—**Cells (5 × 10⁶) were harvested by centrifugation, washed once with Tris-buffered saline, and resuspended to 10⁶ cells/ml in Tris-buffered saline. Binding was carried out in 0.5 ml with N-formyl-Neu-Leu-Phε-Neu-Tyr-Lys-fluorescein at 10 mM. Cells were analyzed on a FACS Calibur flow cytometer (Becton Dickinson) for fluorescent intensity and gated on forward and side scatter to exclude debris and dead cells. Nonspecific binding was determined in the presence of 1 μM N-formyl-Met-Leu-Phe and was almost identical to the level of fluorescence observed with vector-transfected cells.

**Ligand Binding Determinations—**Ligand binding parameters were determined by flow cytometry performed as described above with the following changes. To determine binding parameters (Kᵢ and Bₘₐₓ) more effectively, the higher affinity ligand N-formyl-Neu-Leu-Phε-Neu-Tyr-Lys-fluorescein was used at eight concentrations between 10⁻¹³ and 3.3 × 10⁻¹⁰ M (12). Cell density was decreased to 10⁶ cells/ml to prevent depletion of ligand at low ligand concentrations. Nonspecific binding was determined in the presence of 100 μM N-formyl-Met-Leu-Phe. Binding parameters were calculated using the program Prism (Graphpad). Absolute Bₘₐₓ values were determined by comparing fluorescent intensity with reference beads containing known amounts of fluorescein (Flow Cytometry Standards Corp.).

**Measurement of [Ca²⁺] rm**—Cells were collected by centrifugation and resuspended at 5 × 10⁶ cells/ml in Hanks’ buffered saline solution (HBSS). The cells were incubated with 5 μM indomethacin for 30 min at 37 °C with gentle rocking, washed once with HBSS, and resuspended to a concentration of approximately 10⁶ cells/ml in HBSS containing 1.5 mM EGTA, pH 8.0. The mobilization of intracellular Ca²⁺ concentrations of fMLF was monitored using an SLM 8000 photon-counting spectrofluorometer (SLM-Amino) detecting the ratio of fluorescence at 400 and 490 nm, as described (13). The concentration of intracellular Ca²⁺ was calculated as described (14).

**In Vivo Phosphorylation—**Phosphorylation of the wild type FPR and FPR mutants was determined as described (8). Briefly, FPR-transfected U937 cells were harvested and resuspended in phosphate-free RPMI 1640 containing 1 mM of carrier-free, acid-free [³²P]orthophosphate. Cells were loaded for 3 h at 37 °C and then stimulated with 1 μM fMLF for 10 min at 37 °C. Cells were lysed with radioimmuno precipitation buffer, and insoluble debris was removed by centrifugation. The supernatant was added to 10 mg of protein A-Sepharose coupled with goat anti-FPR antibody and incubated for 1 h while rotating at 4 °C. The beads were serially washed, and bound proteins were eluted with 0.1 M NaOH. Samples were separated by electrophoresis on a 12.5% SDS-polyacrylamide gel, and [³²P]content was determined with a Molecular Dynamics PhosphoImager.

**Desensitization—**Desensitization of calcium mobilization was determined as follows. Cells (1 × 10⁶) were loaded with indo-1/AM as described above for calcium flux determinations and divided into two parts. One was stimulated with 1 μM fMLF for 10 min, whereas the other was treated with buffer only. The cells were then washed three times with HBSS at room temperature to remove surface-bound fMLF and resuspended for assay of calcium mobilization as described above. The response of the treated and untreated cells to a stimulation with 100 nM fMLF was determined. Desensitization was expressed as the percentage decrease of the response of the treated cells relative to the untreated cells.

**Receptor Internalization—**Receptor internalization was determined as the agonist-dependent loss of FPR from the cell surface (15). FPR-transfected U937 cells were harvested, washed, and resuspended in HBSS as above. Cells were then stimulated with 1 μM fMLF for 10 min (or the indicated time) at 37 °C and washed three times with HBSS. Remaining cell surface receptors were determined with 10 nm N-formyl-Neu-Leu-Phε-Neu-Tyr-Lys-fluorescein. Ligand-stained cells were then analyzed for fluorescent intensity on a FACS Caliber flow cytometer with dead cells excluded by a gate on forward and side scatter. Receptor internalization is expressed as the percentage decrease of the cell surface receptors of the treated cells relative to the untreated cells.

**RESULTS**

G protein-coupled receptors are known to undergo rapid phosphorylation following agonist stimulation. Multiple kinases can be involved in these phosphorylation reactions, including members of the G protein-coupled receptor kinase family, which specifically recognize agonist-activated receptors, and second messenger-activated kinases, such as protein kinase A and C (16). Receptor phosphorylation by itself does not appear to prevent coupling to G proteins. Desensitization most likely requires the binding of an accessory molecule, such as arrestin, which specifically binds phosphorylated GPCRs and sterically prevents G protein association (17). Recently, arrestin has also been shown to bind to clathrin, suggesting a mechanism for the concerted desensitization and internalization of GPCRs (18). Whether receptor desensitization and internalization are coupled or independent processes remains largely unknown.

In this report, we characterize the ability of a series of mutant forms of the FPR to undergo phosphorylation, desensitization, and internalization. The mutant receptors used in this study represent variants of the FPR in which Ser and Thr residues have been replaced by Ala or Gly residues (Fig. 1). Mutant ΔST/S319A,T325G,S328A,T329A, T331A,S332G,T334G,T336G,S338G,T339A,S342G lacks all eleven potential phosphorylation sites within the carboxyl terminus and has previously been shown not to undergo either ligand-dependent internalization or desensitization. Mutant ΔA (S328A,T329A,T331A,S332G) and mutant ΔB (T334G,T336G,S338G,T339A) represent two clusters of Ser and Thr residues that each follow acidic residues (Glu326 and Asp333 in the case of mutant ΔA and Asp27 in the case of mutant ΔB) and have previously been shown to be deficient in desensitization. Mutants ΔC (S328A,T329A) and ΔD (T334G,S336G) each alter two of the residues altering significant levels of prostaglandin D₂ production. Last, mutant Δ3 (S319A,T325G,S342G) replaces the three Ser and Thr not collectively replaced in mutants ΔA and ΔB.

Mutant receptors were expressed in the promyelocytic cell line, U937, which, in its undifferentiated state, does not express endogenous FPR but is capable of processing the trans-
fected wild type FPR in terms of phosphorylation, desensitization, and internalization (7, 8, 11). Flow cytometric analysis was used to determine the distribution of receptor-expressing transfectants (Fig. 2). In all cases, greater than 90% of the cells expressed receptor. Quantitative binding analyses using the high affinity FPR ligand N-formyl-Nleu-Leu-Phe-Lys-fluorescein revealed that there were no significant differences in ligand binding affinity between the mutants and the wild type receptor (Table I). Receptor expression levels were found to vary less than 2-fold from the highest (mutant ΔST) to the lowest (mutant ΔST). Receptor-mediated calcium mobilization was also not altered in any of the mutants as compared with the wild type receptor, with an approximately 2-fold range in EC₅₀ values.

We next evaluated the ability of the site-directed mutants of the FPR to undergo phosphorylation when treated with agonist. Previous results and those obtained here indicate that the ΔST mutant does not undergo any significant phosphorylation as compared with the wild type FPR (Fig. 3). Mutants ΔA and ΔB each are phosphorylated at a level corresponding to approximately 50% that of the wild type receptor. Mutants ΔC and ΔD show still greater increases in the level of phosphorylation as Ser and Thr residues are restored to the receptor. Mutant ΔS, which contains all eight Ser and Thr residues in the central portion of the FPR carboxyl terminus, displays phosphorylation similar to that of the wild type FPR. These results suggest that receptor phosphorylation is approximately proportional to the content of potentially phosphorylated residues within the central cluster of eight Ser and Thr residues.

FPR desensitization in response to treatment with fMLF was examined next (Fig. 4, upper panel). Cells were first treated for 10 min with a saturating dose of fMLF, washed free of ligand, and then assayed for calcium mobilization. The wild type receptor displays an approximately 80% reduction in peak calcium mobilization to the second challenge of fMLF. On the contrary, mutant ΔST displays only a 20–25% reduction in calcium mobilization under these assay conditions. Mutant ΔA shows a similar deficit to ΔST, whereas mutant ΔB shows a less pronounced effect. This is consistent with our previous finding that mutant ΔB produces an intermediate level of desensitization. However, mutants ΔC, ΔD, and ΔS demonstrated no defect in their ability to undergo desensitization. The result that mutant ΔS exhibited no defect in desensitization indicates that phosphorylation solely within the remaining eight Ser and Thr residues defined by mutants ΔA and ΔB is sufficient to elicit desensitization. The much greater deficit observed with mutant ΔS as compared with mutant ΔB indicates that phosphorylation of residues Ser³²⁸, Thr³²⁹, Thr³³¹, and/or Ser³³² plays a critical role in the desensitization process, whereas phosphorylation of residues Thr³³⁴, Thr³³⁶, Ser³³⁸, and/or Thr³³⁹ displays a more minor or secondary role. We next examined whether removal of all of the Ser and Thr residues within mutant ΔA was necessary to prevent desensitization. Mutants ΔC and ΔD alter the first and second pair of residues altered in mutant ΔA, respectively. Analysis of these mutants revealed that they desensitized normally, indistinguishably from the wild type receptor. These results indicate that phosphorylation of either site (Ser³²⁸/Thr³²⁹ or Thr³³¹/Ser³³²), in conjunction with the sites defined by mutant ΔB, is sufficient to promote desensitization of the receptor, suggesting redundant sites for FPR desensitization. Furthermore, these results suggest that in the wild type receptor, phosphorylation probably occurs at minimally three sites, defined by ΔC, ΔD, and ΔS.

Receptor internalization has been suggested to represent a possible mechanism for, or at least a contributing factor to, desensitization. In support of this, the mechanisms responsible for receptor desensitization and internalization appear to be intimately associated due to the pivotal role played by arrestin.
were treated with a saturating dose of fMLF. Cells expressing the wild type and mutant forms of the FPR were loaded with indo-1/AM to determine the loss of cell calcium in response to a second exposure to fMLF. Cells expressing the wild type and mutant forms of the FPR were loaded with indo-1/AM prior to analysis by flow cytometry. Remaining cell surface receptor expression (mean fluorescent intensity) is expressed relative to the receptor levels present in cells treated with buffer only. Data shown are representative of 3–5 determinations. 

DISCUSSION

In both these processes. To examine this relationship in the FPR, we next tested the ability of each mutant to be internalized (Fig. 4, lower panel). In the case of the wild type receptor, approximately 80% of the receptor internalizes in 10 min. The ΔST mutant, on the contrary, demonstrates no internalization, indicating an absolute requirement for phosphorylation in this process. However, unlike the profile seen with desensitization, all partially phosphorylated mutants underwent complete internalization, indistinguishable from that of the wild type receptor. To examine whether determining internalization at a fixed time point might be inadequate to resolve differences in the rates of internalization, we determined the time course of FPR internalization for the wild type and mutant receptors. The results indicate that there are no significant differences in the rates of internalization, with half-times for internalization ranging from 3 to 4 min for all mutants including the wild type FPR (Fig. 5). Furthermore, the total levels of internalization are also very similar, varying by no more than 10%. The result that phosphorylation at site B (occurring in mutant ΔA) can support internalization while being inadequate to promote desensitization suggests that distinct mechanisms mediate these two processes. Furthermore, this result demonstrates that FPR internalization cannot be responsible for its desensitization.

FIG. 4. Internalization and desensitization of the wild type and mutant forms of the FPR. Upper panel, FPR desensitization was determined as the decrease in fMLF-stimulated elevation of intracellular calcium in response to a second exposure to fMLF. Cells expressing the wild type and mutant forms of the FPR were loaded with indo-1/AM and stimulated (pretreated) with 1 μM fMLF for 10 min. The cells were then washed extensively to remove bound ligand and reassessed for calcium mobilization in response to a second stimulation with 100 nM fMLF. Data represent mean and S.E. of four experiments. Lower panel, receptor internalization was evaluated by determining the loss of cell surface receptors following exposure to a saturating dose of fMLF. Cells were treated with 1 μM fMLF or buffer only for 10 min at 37 °C, washed extensively, and incubated with 10 nM N-formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys-fluorescein prior to analysis by flow cytometry. Mean fluorescence intensity was taken to represent relative cell surface receptor expression. For both wild type and mutant cells, receptor expression is expressed relative to the receptor present in cells treated with buffer only.

FIG. 5. Time course of internalization of the wild type and mutant forms of the FPR. Receptor internalization was determined as a function of time by determining the loss of cell surface receptors following exposure to 1 μM fMLF. Cells were treated with ligand for the indicated time at 37 °C, and the reaction was quenched with the addition of 4 volumes of ice-cold buffer. The cells were then washed extensively and incubated with 10 nM N-formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys-fluorescein prior to analysis by flow cytometry. Remaining cell surface receptor expression (mean fluorescent intensity) is expressed relative to the receptor levels present in cells treated with buffer only. Data shown are representative of 3–5 determinations. ●, wild type FPR; ○, mutant ΔST; ▷, mutant ΔA; ●, mutant ΔB; △, mutant ΔC; ●, mutant ΔD; △, mutant ΔS.

In support of the conclusion that desensitization and internalization of the FPR occur by distinct mechanisms are results obtained with the m2 muscarinic acetylcholine receptor (m2AChR). This GPCR contains a large third intracellular loop of approximately 180 amino acids, which is known to undergo agonist-mediated phosphorylation (19). In the middle portion of this loop are two clusters of Ser and Thr residues very similar to those in the carboxyl terminus of the FPR. Fig. 6 shows an alignment of the two sequences. The two clusters are designated site A and site B to represent the NH2-terminal and COOH-terminal cluster, respectively. The alignment demonstrates that there is remarkable homology between these two sites in the carboxyl terminus of the FPR and the m2AChR. Within each site, three of the four Ser and Thr residues can be aligned perfectly between the FPR and the m2AChR. Within each site, three of the four Ser and Thr residues can be aligned perfectly between the FPR and the m2AChR, with variation in the position of the Ser and Thr residues. Each cluster is immediately preceded by one or two acidic amino acid residues. The presence of N-terminal acidic residues has been shown to be stimulating factor in the activity of G protein-coupled receptor kinase 2 toward peptide substrates (9). Interestingly, whereas the two sites are directly juxtaposed in the case of the FPR, they are separated by 13 amino acids in the case of the m2AChR. Studies by Pals-Rylaarsdam and Hosey (19) have analyzed the effects of mutations at these homologous sites. Their results demonstrated that mutation of the Ser and Thr residues at site B prevents desensitization, whereas mutation of the Ser and Thr residues at site A desensitized normally. Furthermore, both of these mutants internalized normally. The m2AChR results are similar to ours except for the apparent transposition of sites A and B. In the case of the FPR, mutation of site A prevents desensitization, whereas in the case of the m2AChR, mutation of site B prevents desensitization. Mutation of site B
in the FPR produces a modest inhibition of desensitization, indicating that for maximal FPR desensitization, phosphorylation must take place within both sites A and B. With both mutant receptors, phosphorylation at either site is completely sufficient for internalization to occur.

These results lead to two important questions. First, what structural and functional similarities are there between the FPR and the m2AChR? Second, what are the mechanisms that distinguish between phosphorylation at sites A and B in these receptors? With regard to the first question, similar clusters of Ser and Thr residues exist in numerous other receptors, including the chemotaxtractant receptors for C5a (20) and interleukin-8 (21), which bear high overall homology to the FPR, as well as receptor subtypes for serotonin (22), angiotensin (23), kin-8 (21), which bear high overall homology to the FPR, as well as receptor subtypes for serotonin and Thr residues preceded by acidic residues, involved in directing the desensitization and internalization of GPCRs. Phosphorylation of either of these two clusters is sufficient to mediate internalization, whereas disruption of one cluster can completely inhibit desensitization. These distinctions clearly indicate that these two events, which both require receptor phosphorylation, proceed by different mechanisms. The existence of similar sequences in other GPCR suggests that the results described here may be much more widespread throughout the GPCR superfamily.

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