Phosphorylation of the N-Formyl Peptide Receptor Is Required for Receptor Internalization but Not Chemotaxis*

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The human N-formyl peptide receptor (FPR) is a member of the family of leukocyte, G protein-coupled, chemoattractant receptors. To determine the role(s) of receptor phosphorylation in FPR processing and formylmethionylleucylphenylalanine (fMLF)-mediated chemotaxis, we utilized U937 cells expressing the recombinant wild type receptor and a mutant form of the FPR. This mutant, which lacks all of the serine and threonine residues in the C terminus of the receptor, ΔST, has recently been shown to produce a receptor capable of fMLF binding and G protein activation but was demonstrated not to undergo fMLF-dependent phosphorylation or desensitization of the calcium mobilization response upon repeated exposure to agonist (Prossnitz, E. R. (1997) J. Biol. Chem. 272, 15213–15219). In this report, we examined the role of receptor phosphorylation in FPR internalization and leukocyte chemotaxis. Whereas the wild type receptor was rapidly internalized upon stimulation, the phosphorylation-deficient mutant was not, remaining entirely on the cell surface. In addition, contrary to the hypothesis that receptor processing and recycling are required for chemotaxis, we found no defect in the ability of the mutant FPR to migrate up a concentration gradient of fMLF. These results indicate that phosphorylation of the FPR is a necessary step in receptor internalization but that receptor phosphorylation, desensitization, and internalization are not required for chemotaxis.

Neutrophils normally exist in a resting state as they circulate though the body. However, upon interaction with small molecules known as chemoattractants, they rapidly respond with endothelial adhesion followed by emigration from the vasculature and chemotaxis to the site of inflammation (1). Chemoattractants activate neutrophils through binding to heptahelical receptors located on the cell surface (2, 3). These receptors activate heterotrimeric GTP-binding proteins (G proteins) that initiate numerous elaborate signal transduction cascades, culminating in neutrophil migration and activation. Once at the site of inflammation, neutrophils respond with phagocytosis, superoxide generation, and the release of degradative enzymes (4). One of the most thoroughly studied chemoattractant receptors is the N-formyl peptide receptor (FPR), which recognizes short N-formylated oligopeptides of bacterial or mitochondrial origin (5–7).

Leukocyte chemotaxis has been shown to be dependent on the binding of chemoattractants to their respective receptors (8, 9). Following binding of the ligand and cellular activation, the receptors undergo desensitization and internalization (10, 11). Once internalized, ligand dissociates from the receptor and is degraded, whereupon the receptor is recycled to the cell surface for additional rounds of activation (10). Receptor recycling has been suggested to be essential for sustained cellular responses, such as cell chemotaxis (12, 13). Inhibition of receptor recycling through exposure to wheat germ agglutinin or by neuraminidase treatment was found to block chemotaxis without affecting receptor-mediated superoxide generation or degradation. Receptor endocytosis was also demonstrated to proceed normally with the internalized receptor accumulating within the cell and not being re-expressed to the cell surface (12, 13). A role for neutral endopeptidase has also been implicated through the use of the inhibitor phosphoramidon (14). Treatment with this inhibitor blocked degradation of the internalized ligand as well as re-expression of the internalized receptor, suggesting that dissociation of the ligand from the receptor and its subsequent hydrolysis are essential for receptor recycling (15). In fact, degradation of internalized ligand was shown to occur at a rate proportional to receptor re-expression, suggesting that the former process may be rate-limiting. Inhibition of receptor recycling through this method specifically blocked chemotaxis but not other neutrophil responses, providing further support for the conclusion that receptor recycling is required for chemotaxis (15).

We have recently shown that phosphorylation of the FPR is an essential step in the functional desensitization of the receptor. In this report, we investigated the role of receptor phosphorylation in the internalization process as a means to examine the role of receptor processing in chemotaxis. Our results demonstrate that although receptor phosphorylation is absolutely essential to receptor desensitization and internalization, neither phosphorylation nor receptor internalization is required for cell chemotaxis.

EXPERIMENTAL PROCEDURES

Materials—The cDNA encoding the FPR was obtained from a human HL-60 granulocyte library as described previously (16). N-Formyl-Nle-Leu-Phe-Nle-Tyr-Lys-fluorescein and indo-1AM were obtained from Molecular Probes. fMLF was purchased from Sigma. Carrier-free, acid-free [32P]orthophosphate was from Amersham Corp. Protein A-Sepharose CL-4B beads were obtained from Pharmacia Biotech Inc. Chemotaxis chambers (48-well) were from Neuroprobe with cellulose nitrate filters from Toyo. RPMI was from Whittaker Bioproducts; fetal bovine serum was from HyClone.

Construction and Expression of Site-directed Mutants in U937

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Cells—The FPR cDNA was subcloned and mutagenized as described (16, 17). U937 cells were grown in RPMI 1640 supplemented with 2 mM l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES (pH 7.4), and 10% heat-inactivated fetal bovine serum. For transfection, approximately 4 × 10^6 cells were harvested and resuspended for at least 10 min at 4 °C in HBSS containing 10 mM glucose and 0.1 mM diethiothreitol (18). Linearized DNA (10 μg in a volume of 10 μl) was added to the cells and preincubated for 5 min at room temperature. The cells were then subjected to a 240-V pulse from a 960-microfarad capacitor (resulting in a pulse time constant of approximately 30 ms) and immediately returned to 5–10 ml of culture medium. The following day, G418 was added to a final active concentration of 1 mg/ml. As the selection proceeded, the cells were centrifuged and resuspended in fresh medium (containing G418) at 4–6-day intervals. Cells were cultured at 37 °C in a humidified atmosphere of 6% CO₂ and 94% air.

In Vivo Phosphorylation and Immunoprecipitation—Phosphorylation of the FPR was determined as described (19). Briefly, FPR-transfected U937 cells were harvested and washed extensively to remove traces of phosphate. Cells were resuspended in phosphate-free RPMI 1640 containing 1 mM of carrier-free, acid-free[^32P]orthophosphate (10 mM/cm²). Cells were loaded for 3 h at 37 °C and subsequently stimulated with MFML for 10 min at 37 °C. Cells were lysed by the addition of 0.33 volume of 4 × radioimmunoprecipitation buffer (40 mM Tris-HCl, pH 7.5, 660 mM NaCl, 4 mM EDTA, 0.4% SDS, 2% deoxycholate, 1% Triton X-100, 4 mM p-nitrophenyl phosphate, 40 mM sodium phosphate, 160 mM NaF, 20 mM sodium vanadate, 100 mM leupeptin, 2 mM phenylmethylsulfon fluoride, 400 ng/ml aprotinin, and 200 μg/ml peptatin A). Following lysis, extraction, and removal of insoluble debris, the supernatant was added to 10 μg of Protein A-Sepharose, which had been precoated with 15 μl of a rabbit antiserum directed against the C-terminal 12 amino acids of the FPR, and incubated for 1 h while rotating at 4 °C. The beads were then washed as follows: once with 1 ml of 50 mM Tris-HCl, 500 mM NaCl, 1% Triton X-100, 0.2% SDS, pH 8.0; once with 1 ml of 50 mM Tris-HCl, 500 mM NaCl, 1% Triton X-100, 0.1% SDS, pH 8.0; once with 1 ml of 50 mM Tris-HCl, 500 mM NaCl, pH 8.0; and finally with phosphate-buffered saline. Laemmli sample buffer was added, 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 [^32P] content was determined with a Molecular Dynamics PhosphorImager.

Desensitization—To assess desensitization, the ability of the cells to respond to ligand with a calcium mobilization response was monitored. For calcium determinations, cells were harvested by centrifugation, washed once with phosphate-buffered saline, and resuspended at 5 × 10^6 cells/ml in Hanks’ buffered saline solution (HBSS). The cells were incubated with 5 μM indo-1 AM for 25 min at 37 °C, washed once with HBSS, and resuspended to a concentration of approximately 10^6 cells/ml in HBSS containing 1.5 mM EGTA, pH 8.0. The elevation of intracellular Ca^{2+} by MFML was monitored by continuous fluorescence measurement using an SLM 8000 photon-counting spectrofluorometer (SLM-Amino) detecting at 400 and 490 nm, respectively, as described (16). The concentration of intracellular Ca^{2+} was calculated as described (20). For desensitization determinations, cells were first pre-treated with 1 μM MFML, and the response was recorded. The stimulated cells were then removed from the cuvette, washed three times with HBSS at room temperature to remove surface-bound MFML, and placed in the cuvette. The response of the washed cells to a second stimulation with 1 μM MFML was then determined.

Receptor Internalization—Receptor internalization was first determined as the loss of FPR from the cell surface as follows. FPR-transfected U937 cells were harvested, washed, and resuspended in HBSS. Cells were then stimulated with 1 μM MFML for 10 min at 37 °C and washed three times with HBSS. Remaining cell surface receptors were determined with 10 nM[^3H]F that is internalized and normalized to total fMLF associated with the cell, both internal and external. Free ligand was separated from cell-associated ligand by rapid filtration through glass fiber filters followed by three washes with cold dilution buffer. Results are expressed as the percentage of saturably bound fMLF[^3H] that is internalized and normalized to the amount internalized by the wild type FPR.

Chemotaxis—Cell migration was quantitated with a 48-well chemotaxis chamber (Neuroprobe) using 5.0- or 8.0-μm pore size cellulose nitrate filters (9). Cells and fMLF were prepared in HBSS supplemented with 10 mM HEPES and 1% bovine serum albumin. Chemotactant was placed in the lower chamber and covered with the cellulose nitrate filter. Cells (4 × 10^5/ml) were placed in the upper chamber and incubated at 37 °C for 2 h. Following the incubation, the filter was fixed with isopropyl alcohol, stained with hematoxylin, and mounted on a microscope slide. The distance migrated by the cells (in μm) was determined by the leading edge technique (21). For each evaluation, five fields per duplicate filter were measured at 400-fold magnification. The data are presented as the distance migrated by the leading front of the cells in a 2-h span.

RESULTS AND DISCUSSION

It has been well established that G protein-coupled chemotactant receptors mediate leukocyte chemotaxis (22). The signal transduction cascade initiated by these receptors has also been shown to be essential since treatment of cells with pertussis toxin, which blocks the interaction of G proteins with receptors, completely abolishes leukocyte chemotaxis (23–25). Furthermore, actin polymerization has been demonstrated to be essential, since cytochalasins, which block actin polymerization, also abolish chemotaxis (26, 27). Much of the signaling between these two events, however, remains unclear. We have sought to determine the role of receptor processing in these events. Previous experiments have suggested that recycling of chemotactant receptors is essential for chemotaxis to take place. This conclusion was based on a number of experiments, which correlated a block in the re-expression of internalized chemotactant receptors with a lack of chemotactic ability despite normal ligand binding, cell activation of superoxide generation and degranulation, and normal receptor internalization (15, 28).

In this study, we have used a unique approach to investigate the role of receptor processing in chemotaxis. We have previously demonstrated that U937 myeloid cells stably transfected with chemotactant receptors such as the FPR are capable of migrating up a chemotactic gradient (9). This chemotactic response is indistinguishable from the response observed with differentiated U937, THP-1, or neutrophils. To evaluate the role of receptor processing, we compared U937 cells transfected with the wild type FPR to U937 cells transfected with a mutant form of the FPR, AST, in which all of the serine and threonine residues in the C terminus have been converted to alanines and glycines. Fig. 1 demonstrates that, whereas the wild type FPR becomes phosphorylated upon stimulation with MFML, the AST mutant does not. That the mutant form of the FPR was capable of responding to fMLF was demonstrated by monitoring the ligand bound to the cell surface but has no effect on internalized ligand. Cell samples added to 10 volumes of cold Hank’s buffer (as opposed to pH 3.0 glycine) provided total fMLF[^3H] associated with the cell, both internal and external. Free ligand was separated from cell-associated ligand by rapid filtration through glass fiber filters followed by three washes with cold dilution buffer. Results are expressed as the percentage of saturably bound fMLF[^3H] that is internalized and normalized to the amount internalized by the wild type FPR.
mobilization of intracellular calcium. Upon fMLF stimulation, both the wild type and ΔST mutant underwent similar degrees of calcium mobilization (Fig. 2). This indicated that in the absence of receptor phosphorylation, ligand binding and G protein activation remained intact. We examined desensitization of the fMLF-initiated calcium mobilization response by taking cells that had been exposed to a saturating concentration of fMLF yielding a maximal calcium mobilization response, washing them extensively to remove bound ligand, and determining their responsiveness to a second exposure to fMLF. As expected for the wild type FPR, the amount of calcium mobilization following a second exposure to agonist was minimal (Fig. 2). However, exposing fMLF-treated ΔST cells to a second dose of ligand yielded a calcium response of the same magnitude as that observed for the first exposure. These results confirm that receptor phosphorylation is an essential step in the functional desensitization of the receptor-mediated response.

To determine what mechanism may underlie this phenomenon, we next evaluated the ability of the receptor to undergo ligand-stimulated internalization. Receptor internalization provides possible mechanisms for desensitization by removing occupied receptors from the cell surface to intracellular endosomes. We initially evaluated receptor endocytosis by determining the amount of receptor remaining on the cell surface following a period of exposure to fMLF. Cell surface receptors were quantitated by flow cytometry using N-formyl-Neu-Leu-Phen-Tyr-Lys-fluorescein as a specific probe for cell surface-localized FPR. When U937 cells transfected with the wild type FPR were exposed to fMLF for 10 min at 37 °C and assayed for cell surface receptors, they exhibited a 75% decrease in the number of cell surface receptors compared with cells that had not been exposed to fMLF (Fig. 3A). However, when the same comparison was made with U937 cells expressing the ΔST FPR mutant, which express almost equal numbers of receptors, there was no decrease in the number of cell surface receptors following a pretreatment with fMLF. To confirm that the decrease in cell surface receptors was the result of ligand-mediated receptor internalization, we also measured uptake of tritiated ligand. Increasing concentrations of fMLF[3H]F were incubated with wild type and mutant cells for 1 h at 37 °C. The cells were then transferred to a low pH buffer, which causes dissociation of ligand bound to the cell surface but has no effect on internalized ligand. Wild type FPR-transfected U937 cells demonstrated significant uptake of fMLF[3H]F during the course of the assay (Fig. 3B). On the contrary, cells expressing the ΔST mutant internalized almost no ligand, even at fMLF concentrations 10-fold higher than that required to demonstrate significant uptake with the wild type receptor. These results suggest that receptor phosphorylation is required for internalization as well as desensitization of the FPR and that the two processes may be interdependent.

Having defined a form of the FPR that is capable of binding ligand and initiating signal transduction but incapable of being internalized and thus recycled to the cell surface, we were now able to test the hypothesis that chemotaxis requires recycling of the receptor. Chemotaxis was evaluated using a 48-well chemotaxis chamber with the lower chamber containing chemoattractant separated from the upper chamber containing cells by a 120-μm thick convoluted pore cellulose nitrate filter. This method allows the distinction to be made between simple migration through an “open hole” thin polycarbonate filter and true chemotaxis as revealed with this method. Only myeloid cells are capable of migrating through thick convoluted pore filters whereas many cell types including endothelial cells and fibroblasts, for example, can traverse straight open hole pore filters. The ability of the FPR-transfected U937 cells to undergo
Formyl Peptide Receptor Internalization and Chemotaxis

TABLE I

Checkerboard analysis of FPR-transfected U937 cell chemotactic activity

| FMLF in lower chamber | FMLF in upper chamber |
|-----------------------|-----------------------|
| 0                     | 0                     |
| 1 nM                  | 12                    |
| 10 nM                 | 46                    |
| 100 nM                | 53                    |

Data are expressed as the distance of migration (in microns) in a 2-h period as described under “Experimental Procedures.” Values are means of five measurements on each of duplicate filters and are representative of three experiments.

Chemotaxis mediated by the wild type FPR is independent of receptor phosphorylation. Receptor-mediated chemotaxis was evaluated using a 48-microwell chemotaxis chamber. Cell migration (in μm) was determined as a function of the concentration of FMLF in the lower chamber after an incubation period of 2 h at 37 °C. ∆, wild type FPR-transfected U937 cells; ●, ΔST mutant FPR-transfected U937 cells; ○, vector only-transfected U937 cells. Data are means of duplicate assays, representative of four experiments.

FIG. 4. Chemotaxis mediated by the wild type FPR is independent of receptor phosphorylation. Receptor-mediated chemotaxis was evaluated using a 48-microwell chemotaxis chamber. Cell migration (in μm) was determined as a function of the concentration of FMLF in the lower chamber after an incubation period of 2 h at 37 °C. ∆, wild type FPR-transfected U937 cells; ●, ΔST mutant FPR-transfected U937 cells; ○, vector only-transfected U937 cells. Data are means of duplicate assays, representative of four experiments.

is possible that only in the presence of internalized receptor is receptor re-expression required for chemotaxis to occur. This would indicate that under normal circumstances, replenishing of cell surface receptors is essential to provide the receptors necessary to propagate migration; however, as our results demonstrate, in the absence of receptor depletion through internalization, chemotaxis can be initiated and continue for a prolonged period of time. This demonstrates that signal transduction mediated by a functional chemotactic receptor in the absence of receptor desensitization can control the spatial and temporal aspects of the signal transduction cascade that are involved in the remodeling of the actin cytoskeleton that propels the cell forward during chemotaxis. Although receptor desensitization and internalization are not required for chemotaxis, these processes are involved in preventing chronic activation of leukocytes at sites of inflammation. To compensate for the possible lack of receptors during the relatively long periods of chemotaxis, it appears that re-expression of internalized receptors has evolved as a mechanism to ensure sufficient cell surface receptors. In conclusion, although receptor recycling occurs during chemotaxis, it is not an essential component of the chemotactic phenomenon.

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