Articles

Multiple Activation Steps of the *N*-Formyl Peptide Receptor[†]

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ABSTRACT: The human *N*-formyl peptide receptor (FPR) is representative of a growing family of G protein-coupled receptors (GPCR) that respond to chemokines and chemoattractants. Despite the importance of this receptor class to immune function, relatively little is known about the molecular mechanisms involved in their activation. To reveal steps required for the activation of GPCR receptors, we utilized mutants of the FPR which have previously been shown to be incapable of binding and activating G proteins. For this study, the FPR mutants were expressed in human myeloid U937 cells and characterized for functions in addition to G protein coupling, such as receptor phosphorylation and ligand-induced receptor internalization. The results demonstrated that one of the mutants, R123G, though being unable to activate G protein, was capable of undergoing ligand-induced phosphorylation as well as internalization. Receptor internalization was monitored by following the fate of the ligand as well as by directly monitoring the fate of the receptor. The results with the R123G mutant were in contrast to those obtained for mutants D71A and R309G/E310A/R311G which, though being expressed at the cell surface and binding ligand, were incapable of being phosphorylated or internalized upon agonist stimulation. These results suggest that following ligand binding at least two "steps" are required for full activation of the wild-type FPR. That these observations may be of more general importance in GPCR-mediated signaling is suggested by the highly conserved nature of the mutants studied: D71, R123, and the site represented by amino acids 309−311 are very highly conserved throughout the entire superfamily of G protein-coupled receptors. Models of receptor activation based on the observed results are discussed.

Cellular activation usually occurs through the binding of agonists to cell surface membrane receptors followed by the generation of intracellular second messengers. The largest class of these receptors belongs to the family of GTP-binding regulatory protein-coupled receptors. This superfamily, consisting of hundreds to thousands of members, is exemplified by its membrane topology of seven membrane-spanning domains. Transmembrane signaling is initiated by the binding of agonist to the extracellular surface or transmembrane region of the receptor. Agonist binding is postulated to induce a conformational change in the receptor resulting in the catalytic activation of heterotrimeric G proteins.[1] Despite the abundance of these receptors, relatively little is known regarding the mechanisms involved in the activation of the receptor by agonist as well as the subsequent mechanisms of G protein activation. A model for the activation of G protein-coupled receptors has been described and is referred to as the ternary complex model (*1*). This model describes the signaling competent form of a receptor as a ternary complex, resulting from the sequential association of ligand and G protein with receptor. Recent experiments have revealed that this model is inadequate to explain the characteristics of a class of mutant adrenergic receptors that display activity in the absence of ligand (*2*). Studies with these receptors have suggested that the unliganded wild-type receptor exists in an equilibrium between active and inactive conformations with the inactive conformation being pre-

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[1] Abbreviations: FPR, *N*-formyl peptide receptor; fMLF, *N*-formyl-methionyl-leucyl-phenylalanine; G protein, guanine nucleotide-binding regulatory protein.

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dominant in the absence of agonist. Only the active form of a receptor, which is stabilized by agonist, is capable of initiating signal transduction.

Another G protein-coupled receptor which has been used as a model for understanding the activation mechanisms of receptors is the chemoattractant *N*-formyl peptide receptor (FPR) of neutrophils (*3*). Many chemoattractant receptors have recently been cloned, including the receptors for *N*-formyl peptides, complement components C_{3a} and C_{5a}, IL-8, and numerous chemokine receptors (*4*). These receptors are all members of the class of G protein-coupled receptors, appearing to activate predominantly pertussis toxin-sensitive G protein subtypes (*5*). Studies of the FPR have revealed that the ligand-binding pocket of the receptor is large enough to accommodate formylated peptides up to about 5 amino acids in length (*6*) and that receptor residues at the base of the first and third extracellular loops appear to be involved in ligand recognition (*7*, *8*). Following ligand binding, the FPR activates G proteins predominantly through its second intracellular loop and carboxy terminus (*9*). The third intracellular loop of the receptor, which has been shown to be important in G protein activation in adrenergic and muscarinic receptor systems, appears not to play a significant role in activation by the FPR (*10*). Phosphorylation of the FPR carboxy terminus has been demonstrated following ligand stimulation and has recently been shown to be required for desensitization of cellular responsiveness to fMLF (*11*) but was subsequently demonstrated not to be required for chemotaxis (*12*). Spectrofluorometric determinations using fluorescent formyl peptides have suggested that the assembly of the FPR ternary complex is rapid, occurring with a half-time of much less than a second, consistent wtih the proposal that a portion of the receptor population may be precoupled to G protein (*13*−*15*).

In this study, we describe the characterization of FPR mutants in the second transmembrane domain, the second intracellular loop, and the carboxy-terminal domain which initially appeared to exhibit a common defect in receptor activation. More detailed analyses, however, revealed that the mutants represented blocks in activation at two distinct steps, one required for "receptor activation", the other specifically for G protein activation by the receptor. The results have important implications for our understanding of the mechanisms of receptor activation and signal transduction.

MATERIALS AND METHODS

Materials. fMLF was purchased from Sigma. [^{3}H]fMLF was from Du Pont-New England Nuclear. *N*-Formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys-fluorescein and indo1-AM were obtained from Molecular Probes. Pertussis toxin was from List Biological Laboratories. RPMI 1640 was from Whittaker Bioproducts. Fetal bovine serum was from HyClone.

Construction and Expression of Site-Directed Mutants in U937 Cells. The FPR gene was mutagenized as previously described (*10*). For transfection, U937 cells (4×10^6) 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) were harvested and resuspended in 400 μL of RPMI 1640 containing 10 mM glucose and 0.1 mM dithiothreitol (*16*). Linearized DNA (10 μg) was added and the cells were electroporated with a 240 V pulse from a 960 μF capacitor and returned to 5 mL of growth media. For selection, G418 was added to a final active concentration of 1 mg/mL. Cells were cultured at 37 °C in a humidified atmosphere of 6% CO_2 and 94% air.

Flow Cytometry. Cells were harvested by centrifugation, washed once with PBS, and resuspended to 10^6 cells/mL in PBS. Binding was carried out in 0.5 mL with *N*-formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys-fluorescein at 10 nM. Cells were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) 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.

Ligand Dissociation. U937 cells were harvested by centrifugation and resuspended at 10^6 cells/mL in extracellular buffer (0.11 M NaCl, 30 mM HEPES, 10 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 0.1% (w/v) BSA, 1 mM PMSF, 200 units/mg catalase, 0.05 mg/mL chymostatin, and 320 units/mg superoxide dismutase) and incubated with 5 mg/mL digitonin (Calbiochem, San Diego, CA). Cells were incubated for 30 min at 37 °C with occasional gentle agitation, washed once to remove detergent, resuspended at 10^6 cell/mL in binding buffer (0.1 M KCl, 20 mM NaCl, 1 mM EGTA, 30 mM HEPES, 1 mM PMSF, 1 mM $MgCl_2$, 0.1% (w/v) BSA), and placed on ice. Dissociation of ligand from the cells was determined as follows. Aliquots (0.5 mL) of cells at 10^6/mL were incubated with *N*-formyl-Met-Leu-Phe-Lys-fluorescein at a final concentration of 10 nM for 20 min at 37 °C. Dissociation of peptide was initiated by the addition of 10^{-5} M final concentration blocking peptide (*N*-formyl-Met-Leu-Phe-Lys) in 0.5 mL of binding buffer. Fluorescence intensity was monitored in real-time on a FACScan flow cytometer by setting time as a parameter versus green (FITC) fluorescence (FL1). Addition of the blocking peptide required removing the sample tube from the cytometer while data was acquiring and introduced a lag of no more than 10 s into the data traces. Traces were measured until the fluorescence intensity plateaued or for a maximum of 8.5 min. Cytometric dot plots of fluorescence intensity (FL1) versus time were converted to mean channel fluorescence versus time using a software program developed by Larry Seamer (University of New Mexico). Data compilation and nonlinear regression were performed on the software package Prism (Graphpad Software, San Diego, CA) using single or double exponential decay equations.

Determination of $[Ca^{2+}]_i$. Cells were collected by centrifugation and resuspended at 5×10^6 cells/mL in Hank's 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 mobilization of intracellular Ca^{2+} by the indicated concentration of fMLF was monitored using an SLM 8000 photon-counting spectrofluorometer (SLM-Aminco) detecting at 400 and 490 nm, as described (*17*). The concentration of intracellular Ca^{2+} was calculated as described (*18*).

In vivo Phosphorylation. U937 cells were grown to a density of $1.0-1.5 \times 10^6$ cells/mL and washed three times with 150 mM NaCl, 10 mM HEPES, pH 7.4, to remove

traces of inorganic phosphate prior to phosphate loading. Cells (5×10^6) were resuspended in phosphate-free RPMI-1640 containing 10 mM HEPES, pH 7.4, to a volume of approximately 0.5 mL and loaded for 3 h at 37 °C with 1 mCi carrier- and acid-free [^{32}P]orthophosphate (10 mCi/mL). Following loading, cells were stimulated as indicated and lysed by the addition of 0.33 vol 4x RIPA 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 PMSF, 400 ng/mL aprotinin, and 200 μg/mL pepstatin A. After extraction (10 min, 4 °C), samples were centrifuged at 15000*g* for 15 min at 4 °C to remove insoluble debris. The resulting supernatant was added to Protein A Sepharose (10 mg) 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 PBS. Laemmli sample buffer was added, and the samples were heated at 37 °C for 10 min. Electrophoresis was carried out on a 12.5% SDS−polyacrylamide gel. Gels were dried and relative determinations of ^{32}P content were performed with a Molecular Dynamics Phosphorimager.

Receptor Internalization. Internalization of fML[^{3}H]F was determined as follows. FPR-transfected U937 cells were harvested, washed, and resuspended in Hanks balanced salts solution (HBSS). Cells were incubated with the indicated concentration of fML[^{3}H]F at 37 °C for 1−120 min. Control cells were incubated on ice (which prevents endocytosis) or with excess unlabeled fMLF to determine nonspecific binding and internalization. After incubation, cell samples were added to 10 volumes of 0.2 M glycine (pH 3.0) containing 0.5 M NaCl for 5 min on ice. This incubation removes ligand bound to the cell surface but has no effect on internalized ligand. Cell samples added to 10 volumes of cold Hanks buffer (as opposed to pH 3.0 glycine) provided total fML[^{3}H]F 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 buffer. Results are expressed as the percentage of saturably bound fML[^{3}H]F that is internalized and normalized to the amount internalized by the wild-type FPR.

In addition to measuring intracellular ligand accumulation, receptor internalization was also determined as the loss of FPR from the cell surface as follows. FPR-transfected U937 cells were harvested, washed, and resuspended in HBSS as above. Cells were then stimulated with 1 μM fMLF for 10 min at 37 °C and washed three times with HBSS. Remaining cell surface receptors were determined with 10 nM *N*-formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys-fluorescein or a monoclonal antibody (5F1−9, Leukosite Inc., see below) directed against the extracellular portions of the FPR. In the latter case, after a wash to remove unbound primary antibody, cells were subsequently incubated with fluorescein-conjugated goat anti-mouse antibodies. Ligand- or antibody-stained cells were then analyzed for fluorescent intensity on a FACScan flow cytometer with dead cells excluded by a gate on forward and side scatter. Receptor internalization was determined relative to cells which had not been treated with fMLF.

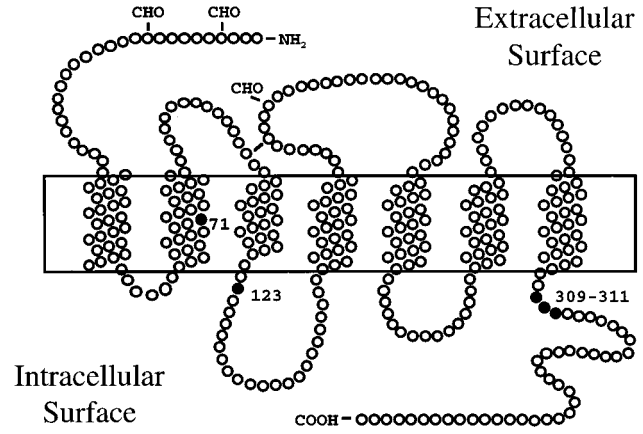

FIGURE 1: Schematic representation of the FPR and the locations of site-directed mutants. Locations of the site-directed mutants are indicated by solid circles and the amino acid number(s) of the mutant: Asp71, Arg123, and Arg309/Glu310/Arg311.

Generation of anti-FPR Monoclonal Antibody. Monoclonal antibodies were made to the FPR using as an antigen the Abelson-transformed L1/2 lymphoma cell line transfected with the human FPR cDNA (*19*). The cDNA for the human FPR was amplified from peripheral blood using PCR primers designed from Genbank accession number M60626, and directionally cloned into the expression vector pMRB-101 (selectable with mycophenolic acid). Linearized plasmid was transfected into L1/2 cells by electroporation as previously described (*20*, *21*). After drug selection, the highest expressors were enriched by several rounds of fluorescence-activated cell sorting of transfectants stained with *N*-formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys-fluorescein. The resulting population expressed ~50−100 thousand receptors per cell (*20*). C57BL/6 mice were immunized ip with FPR-transfected L1/2 cells at two week intervals. The last immunization was given iv, and 3 days later, one mouse was sacrificed and spleen cells were fused with SP2/0 cells (*22*). Positive clones were identified by flow cytometry through staining of the FPR L1/2 transfectant and human neutrophils, and the specificity against the FPR was confirmed through negative staining with a series of L1/2 transfectants expressing various other G-protein-coupled receptors. One of the clones, designated 5F1-9, was subcloned by limiting dilution and used in this study.

RESULTS

G protein-coupled receptors activate a large number of intracellular effectors through their interactions with heterotrimeric G proteins. To gain a better understanding of the mechanisms involved in receptor activation, we have further characterized mutants of the FPR that were previously demonstrated to be unable to bind G protein in vitro or activate G proteins in vivo (*23*). The mutants studied were D71A, located in the second transmembrane segment, R123G, located at the boundary of the third transmembrane domain and the second intracellular loop, and R309G/E310A/R311G, located at the beginning of the carboxy-terminal domain (Figure 1). These residues represent sites of significant conservation between G protein-coupled receptors. In particular, R123, part of the "DRY" consensus sequence at

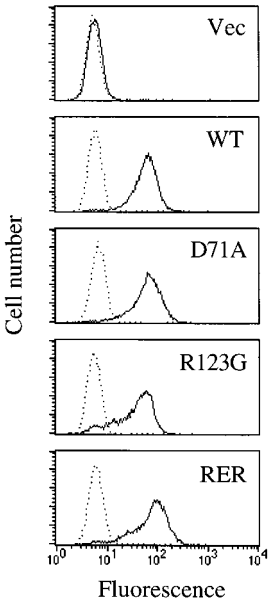

FIGURE 2: Cell surface expression of the wild-type and mutant forms of the FPR. Expression was evaluated for cells transfected with vector only as a control, the wild-type FPR, and each mutant FPR [D71A, R123G, and RER/GAG (denoted RER)] by flow cytometry, plotting cell number versus fluorescent intensity. Binding was determined with *N*-formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys-fluorescein at 10 nM. Solid and dotted lines represent binding in the absence and presence of 10 μM fMLF, respectively. Data are representative of three experiments.

the beginning of the second intracellular loop, is conserved in all G protein-coupled receptors. When characterized in the mouse fibroblast cell line, L2071, these mutant forms of the FPR demonstrated defective binding to and therefore activation of G proteins. We proposed at that time that the mutant forms of the FPR may stabilize the inactive conformation of the receptor, thus preventing its activation in the presence of ligand. An alternate hypothesis was that the mutations occurred at sites of direct interaction between the receptor and G protein thus preventing binding and activation of the G protein. In this latter case, G protein-independent functions of the receptor might be unaffected by the mutation.

For the purposes of this study, mutant forms of the FPR were stably expressed in a native environment, the human myeloid cell line, U937. This cell line does not express the FPR in its undifferentiated state, but can be induced to express numerous chemoattractant receptors upon differentiation into a more mature myeloid cell, with agents such as dibutyryl-cAMP or PMA (*24*). Cell surface expression of the mutant forms of the FPR was assessed by flow cytometry with the ligand *N*-formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys-fluorescein (Figure 2). Cells transfected with the vector alone were used as a control, whereas non-specific binding was determined in the presence of excess fMLF (Figure 2, dotted lines). Vector-transfected U937 cells displayed no specific binding of the fluorescent ligand; however, the wild-type and mutant forms of the FPR all displayed substantial and similar levels of cell surface receptor expression. This binding was completely blocked in the presence of 10 μM fMLF. G protein interaction with the receptor was evaluated spectrofluorometrically by measuring the dissociation rate of 10 nM *N*-formyl-Met-Leu-Phe-Lys-fluorescein from digitonin permeabilized wild-type and mutant receptors in the presence and absence of the nonhydrolyzable GTP analogue, GTPγS. In the absence of added guanine nucleotide, receptors exist in two states, ternary ligand−receptor−G protein complex and binary ligand−receptor complex, which can be distinguished by the dissociation rates of ligand from the receptor. The active ternary ligand−receptor−G protein complex exhibits a slow ligand dissociation rate, reflecting a high affinity for ligand. In the presence of guanine nucleotide, the activated ternary complex destabilizes, the G protein dissociates from the receptor, and the dissociation of only the low-affinity ligand−receptor complex is measured (*13*, *15*, *25*). The wild-type receptor in the absence of GTPγS (Figure 3a, upper curve) demonstrated dissociation kinetics consisting of two components, 0.07 and 0.006 s^{-1}, representing the ligand−receptor and ligand−receptor−G protein complexes, respectively. However, in the presence of GTPγS (Figure 3a, lower curve) a single dissociation rate, 0.12 s^{-1}, was observed, consistent with only a single species, ligand−receptor, being present. Similar to the wild-type receptor in the presence of GTPγS, the mutant receptors, in the absence of GTPγS, also demonstrated only the ligand−receptor complex to be present (single dissociation rates between 0.04 and 0.11 s^{-1}). In addition, there was no alteration in their ligand dissociation kinetics in the presence of GTPγS (Figure 3b, c and d), further indicating that they were not interacting with G protein.

The functional capabilities of the mutant forms of the FPR, expressed in U937 cells, were evaluated by measuring the ligand-stimulated rise in intracellular calcium with the fluorescent indicator indo-1 (Figure 4). Whereas the wild-type FPR yielded a dose-dependent rise in intracellular calcium in response to fMLF, none of the mutants were capable of mediating a calcium response, even at fMLF concentrations 1000-fold higher than that required to yield a response for the wild-type receptor. These results were consistent with the kinetic binding data and confirmed our previous results from transfected L cells that these mutations affect G protein coupling. However, based on the existing data, the mechanism responsible for the defect in activation of G proteins could not be determined.

To test the possibility that functions other than G protein activation of the FPR might be unaffected in these mutants, we first employed the fact that G protein-coupled receptor phosphorylation has been shown to be independent of G protein activation (*26*). We have recently demonstrated that the FPR undergoes ligand-dependent phosphorylation in U937 cells, consistent with the idea that phosphorylation of G protein-coupled receptors requires the same conformational change in the receptor that is required for G protein activation (*11*). Ligand-dependent phosphorylation of the wild-type and mutant forms of the FPR was assessed in [^{32}P]orthophosphate-labeled, transfected U937 cells by immunoprecipitation of the fMLF-stimulated FPR (Figure 5). Compared to the wild-type FPR, mutants D71A and RER/GAG demonstrated no significant receptor phosphorylation. However, the R123G mutant demonstrated significant phosphorylation approaching 50% of the level observed with the wild-type receptor. In all cases, there was no phosphorylation in the absence of ligand (data not shown). This result suggested that the liganded R123G mutant was capable of being recognized by a receptor kinase, whereas the liganded D71A and RER/GAG mutants were not.

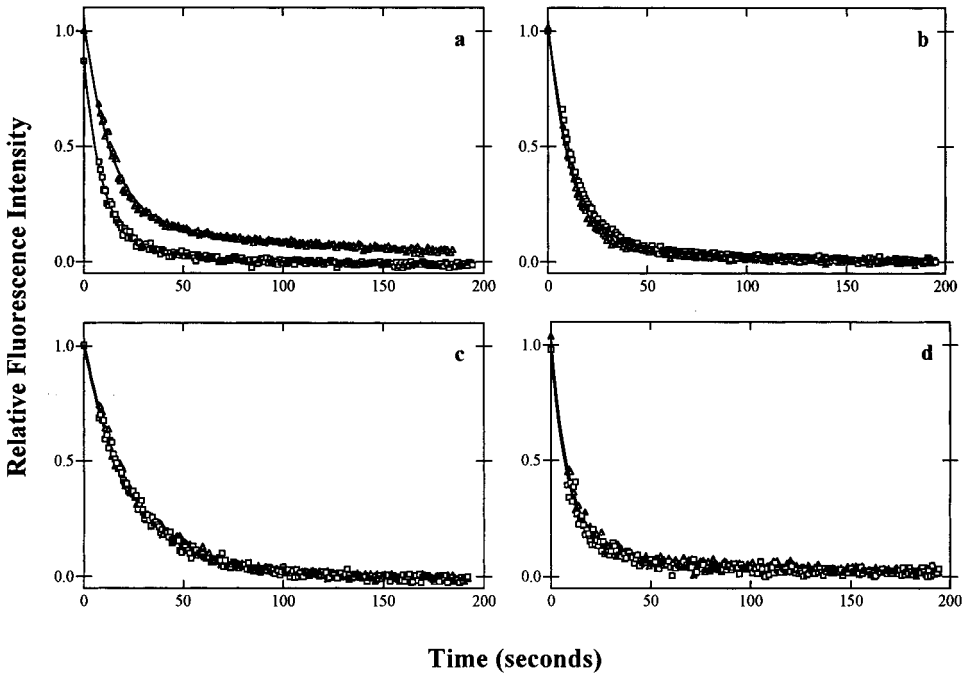

FIGURE 3: Fluorescent formyl peptide dissociation from permeabilized wild-type and mutant forms of the FPR. Normalized dissociation of 10 nM *N*-formyl-Met-Leu-Phe-Lys-fluorescein over time from permeabilized wild-type FPR (a), RER (b), R123G (c), and D71A (d) in the presence (□) and absence (△) of GTPγS. Peptide dissociation was accomplished by the addition of a saturating concentration of *N*-formyl-Met-Leu-Phe-Phe-Gly-Gly-Lys (10^{-5} M) and a 2-fold dilution in binding buffer. Data are representative of three experiments.

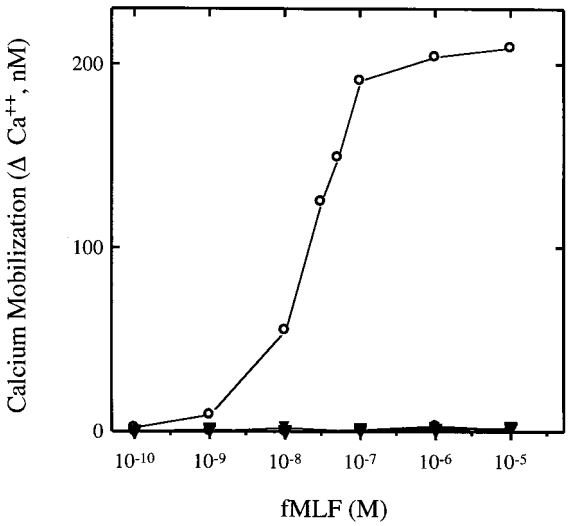

FIGURE 4: Calcium mobilization of the wild-type and mutant forms of the FPR. fMLF-stimulated elevation of intracellular calcium was determined in cells expressing the wild-type (○) and mutant forms of the FPR [D71A (●), R123G (◆), and RER/GAG (▼)]. Cells were loaded with indo-1AM and stimulated with the indicated concentration of fMLF. Data are representative of four experiments.

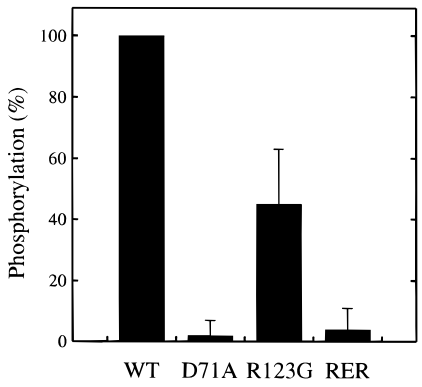

FIGURE 5: Phosphorylation of the wild-type FPR and mutant forms of the FPR. Wild-type and mutant FPR-transfected, [^{32}P]orthophosphate-loaded U937 cells were stimulated with 1 μM fMLF and incubated at 37 °C for 10 min. The FPR was solubilized, immunoprecipitated, and analyzed for its degree of phosphorylation following separation on a 12% SDS−polyacrylamide gel and imaging on a Molecular Dynamics phosphorimager. Data shown are means of three experiments ±SE.

To determine, through another independent mechanism, whether the R123 mutant was capable of being recognized by cellular machinery as an active conformation, we examined the ability of the FPR to undergo ligand-induced internalization. Since the FPR mutants do not activate G proteins, it was first necessary to establish that internalization of the wild-type receptor was not dependent on intracellular signaling initiated by G proteins. Wild-type FPR-transfected U937 cells were treated overnight with pertussis toxin to block G protein activation by ADP ribosylation of the α subunit and assessed for uptake of fML[^{3}H]F (Figure 6). Calcium mobilization was assessed as above to determine the effectiveness of the pertussis toxin treatment (Figure 6, inset). Although pertussis toxin treatment completely abolished calcium mobilization and therefore G protein activation, it had no effect on the internalization of the liganded FPR. This indicated that ligand-induced internalization occurs in the absence of G protein activation but instead is dependent upon the ability of cellular machinery to recognize the active conformation of the receptor. We next examined the ability of the mutant forms of the FPR to mediate uptake of fML-[^{3}H]F as a function of ligand concentration (Figure 7). Whereas the wild-type FPR demonstrated internalization at all ligand concentrations measured, the D71A mutant and, to a similar extent, the RER/GAG mutant did not internalize, even at substantially higher concentrations of ligand. However, as was observed with receptor phosphorylation, the

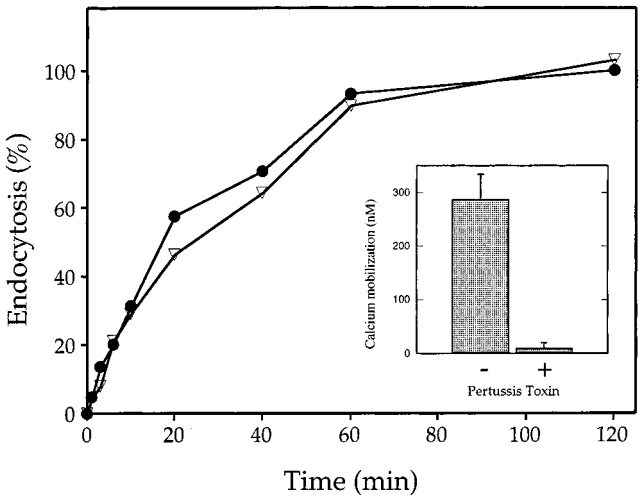

FIGURE 6: Internalization of the FPR does not require G protein activation. FPR-transfected U937 cells were treated with pertussis toxin (100 ng/mL for 16 h under growth conditions, ▽) or buffer only (●). Cells were then harvested and incubated with 20 nM fML-[^{3}H]F at 37 °C for 1−120 min. Control cells were incubated on ice or with excess unlabeled fMLF to determine nonspecific uptake. After incubation, free ligand was separated from internalized ligand by low pH and filtration. Results are expressed as the percentage of saturably bound fML[^{3}H]F that is internalized and normalized to the amount internalized by the untreated FPR. (Inset) Peak mobilization of intracellular calcium in response to 1 μM fMLF by pertussis toxin-treated and control cells, as described in Materials and Methods. Data shown are means of duplicate determinations and representative of three experiments.

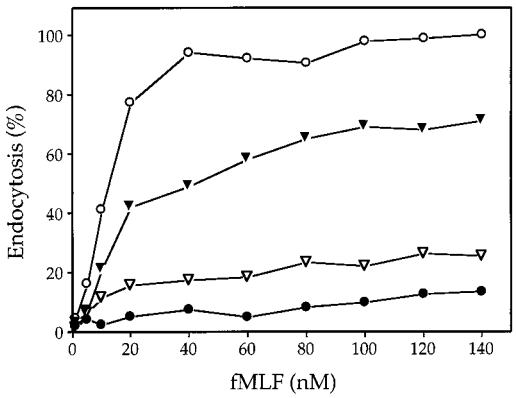

FIGURE 7: Receptor-mediated uptake of fMLF by wild-type and mutant forms of the FPR. Wild-type FPR- or mutant FPR-transfected cells were incubated with the indicated concentration of fML[^{3}H]F at 37 °C for 60 min. Receptor expression for each mutant was determined by incubation with 100 nM fML[^{3}H]F on ice for 60 min, followed by filtration. Ligand uptake for wild-type receptor (○), R123G (▼), RER/GAG (▽), and D71A (●) was normalized to the level of receptor expression, and these values were then normalized to the maximal uptake observed for the wild-type FPR. Data shown are representative of five experiments.

R123 mutant underwent apparent "conformational alterations" as evidenced by the ligand-induced internalization of this mutant FPR. The level of internalization of the R123 mutant approached 70% of the wild-type FPR.

As an alternate method to measuring intracellular ligand accumulation, we also quantitated receptor internalization by measuring the amount of cell surface receptor present before and after exposure to agonist. Cells were exposed to a high concentration of unlabeled fMLF (1 μM) or vehicle only for 10 min at 37 °C, then washed extensively to remove unbound as well as bound ligand. Cells were then incubated with either

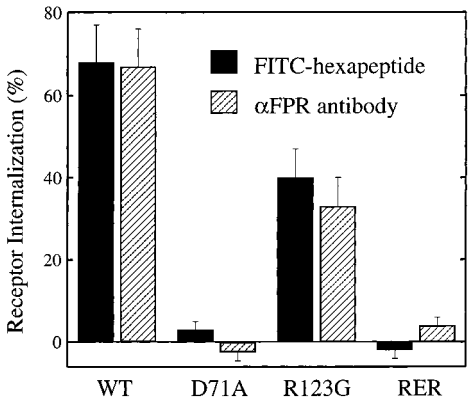

FIGURE 8: Agonist-dependent internalization of the mutant receptor forms of the FPR. Wild-type FPR- and mutant FPR-transfected U937 cells were incubated with 1 μM fMLF at 37 °C for 10 min. The cells were then extensively washed and stained with either *N*-formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys-fluorescein (solid bars) or anti-FPR antibody 5F1−9 (hatched bars) and analyzed by flow cytometry for fluorescence intensity. In each instance, fluorescence was compared to control cells which had not been exposed to fMLF. Data shown are means of three experiments ±SE.

the fluorescent ligand *N*-formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys-fluorescein or a monoclonal antibody (5F1-9), reactive against extracellular portions of the FPR, followed by a fluorescein-labeled second antibody. Total fluorescence intensity was analyzed by flow cytometry for cell surface receptor levels. The results for both methods were consistent and demonstrated that approximately 70% of the wild-type FPR was internalized in 10 min under these conditions (Figure 8). The D71A and RER/GAG mutants demonstrated no decrease in the number of cell surface receptors after the fMLF treatment, indicating a complete lack of receptor internalization. On the contrary, the R123G mutant underwent a significant decrease in the number of cell surface receptors present following the fMLF exposure, confirming internalization of this mutant FPR.

DISCUSSION

In the current study, we have begun to gain a more thorough understanding of the mechanisms involved in the activation of a representative chemoattractant G protein-coupled receptor, the FPR. Previous studies of other GPCRs have identified sites within receptors which are capable of dissociating agonist binding from functional signal transduction (i.e., G protein activation), but in these studies, the mechanism responsible for the defect was not described (*27*−*29*). We have taken two approaches to define, at a molecular level, the steps involved in receptor activation and signaling. First, having described mutant forms of the FPR that are unable to activate G protein-mediated functions, we used physical reconstitution of receptor and G protein to demonstrate that the cause behind this defect was an inability of the agonist-occupied receptor to bind G protein. These previous findings led to the proposal that the mutant forms of the receptor may represent a ligand-bound inactive form of the receptor that is incapable of binding G protein.

To determine whether the defect(s) responsible for the lack of G protein activation represent(s), as suggested above, a global defect in the ability of the receptor to assume a native conformation or alternatively represent a specific defect in a site or sites of direct G protein interaction with the receptor,

we evaluated several G protein-independent functions of the receptor. Two functions of the FPR, which are independent of G protein activation but require agonist binding, are the ability to be phosphorylated by a G protein-coupled receptor kinase and the ability to undergo internalization. Both of these assays revealed that the previously described mutants could be divided into two categories: (1) D71A and RER/GAG, despite binding ligand, were essentially inactive in all assays tested; and (2) R123G was unable to activate G protein but demonstrated agonist-mediated receptor phosphorylation and internalization. Thus it can be concluded that the described mutants identify two distinct steps in the conformational regulation of a G protein-coupled receptor. Other explanations are of course also possible. For example, the D71A and RER/GAG mutants could each represent sites that are essential to all three functions: G protein activation, receptor phosphorylation, and receptor internalization. Though this may be possible for the RER/GAG mutant, which is located at the intracellular boundary of the seventh transmembrane domain, it is less likely for the D71A mutant, which is located close to the middle of the second transmembrane domain. The D71A mutant is likely exerting its effect at a distance, most likely by preventing the receptor from attaining an active conformation, in essence exerting its effect on multiple effector pathways through a single mechanism.

On the other hand, the R123G mutant exhibited a very selective deficiency. Its inability to activate G proteins contrasted its ability to be phosphorylated and internalized. This indicated that the mutant was indeed able to assume the active conformations necessary to be recognized by the receptor kinase and internalization machinery. Only the region, or one of the regions, selectively recognized by G proteins is affected in the R123G mutant. Since phosphorylation and internalization require the wild-type receptor to be agonist-activated, the results suggest that the ligand-bound conformation of the R123G mutant is similar to that of the ligand-bound conformation of the wild-type receptor. The site of the R123G mutant may thus represent an essential contact site between activated receptor and G protein. These results suggest the model shown in Figure 9. The ligand-free receptor exists in an inactive conformation, either in an isolated form or precoupled to G protein. In either case, upon binding ligand, a conformational change takes place exposing sites within the intracellular regions of the wild-type receptor causing G protein interaction and activation. In the case of the D71 mutant, this conformational change is blocked and the intracellular sites that signal receptor activation are not exposed. Some of these sites, such as the RER site at the beginning of the carboxy-terminal tail, may either be recognized by multiple effectors (G proteins, kinases) or may block activation in a manner similar to that of D71A, whereas others, such as R123, are specific for only one aspect of receptor function. Thus, presentation of R123 in the wild-type receptor may represent the critical event in the activation of G proteins. The absolute conservation of an arginine at this position speaks to the potential importance of this residue in signaling.

Numerous studies have examined the effects of mutation within the highly conserved DRY sequence. For the most part, these reports have indicated that the arg residue in the DRY sequence is critical for G protein activation. Examples include the m1 muscarinic receptor (*30*), V2 vasopressin receptor (*29*), and rhodopsin (*31*). Ligand-mediated conformational changes occurring through transmembrane region 3 are likely transmitted to the second intracellular loop. These conformational changes are likely to expose the arg residue of the DRY sequence to the cytoplasmic environment from its usually buried location toward the interior of the receptor (*32*, *33*). Our results extend these observations in that only the G protein-coupling function of the R123G mutant is lost, leaving phosphorylation and internalization intact, suggesting that the conformational change required to expose R123 occurs, but due to the lack of the required amino acid at that position, cellular responses are incomplete.

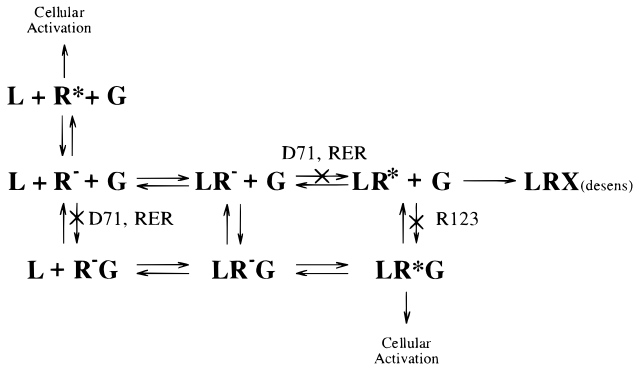

FIGURE 9: Model of FPR activation and the steps blocked by site-directed mutants. In this model for receptor activation, inactive receptor (R^-) binds ligand (L), which leads to the formation of a transient intermediate (LR^-) which is also inactive and unable to interact productively with G protein. This state then converts to an active ligand−receptor complex (LR*), which interacts with and activates G proteins (G), leading to cellular activation. An alternate path involving the binding of ligand to precoupled receptor-G protein complexes (R^-G) is also depicted. The R123G mutant is represented as blocking the ability of the ligand−receptor complex LR* from binding to and activating G protein but not from being phosphorylated and internalized, generating LRX(desens). The D71A mutant is depicted as blocking an upstream common step, the conversion of the inactive LR^- form to that active LR* as well as the conversion of R^- to R^-G. The RER/GAG mutant may either block a common activation step as does D71A (as depicted) or represent a common intracellular site involved in the conversion to LRX(desens) as well as to LR*G.

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