Functional Capabilities of an *N*-Formyl Peptide Receptor−$G_{\alpha i2}$ Fusion Protein: Assemblies with G Proteins and Arrestins†

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ABSTRACT: G protein-coupled receptors (GPCRs) must constantly compete for interactions with G proteins, kinases, and arrestins. To evaluate the interactions of these proteins with GPCRs in greater detail, we generated a fusion protein between the *N*-formyl peptide receptor and the $G_{\alpha i2}$ protein. The functional capabilities of this chimeric protein were determined both in vivo, in stably transfected U937 cells, and in vitro, using a novel reconstitution system of solubilized components. The chimeric protein exhibited a cellular ligand binding affinity indistinguishable from that of the wild-type receptor and existed as a complex, when solubilized, containing $\beta\gamma$ subunits, as demonstrated by sucrose density sedimentation. The chimeric protein mobilized intracellular calcium and desensitized normally in response to agonist. Furthermore, the chimeric receptor was internalized and recycled at rates similar to those of the wild-type FPR. Confocal fluorescence microscopy revealed that internalized chimeric receptors, as identified with fluorescent ligand, colocalized with arrestin, as well as G protein, unlike wild-type receptors. Soluble reconstitution experiments demonstrated that the chimeric receptor, even in the phosphorylated state, existed as a high ligand affinity G protein complex, in the absence of exogenous G protein. This interaction was only partially prevented through the addition of arrestins. Furthermore, our results demonstrate that the GTP-bound state of the G protein α subunit displays no detectable affinity for the receptor. Together, these results indicate that complex interactions exist between GPCRs, in their unphosphorylated and phosphorylated states, G proteins, and arrestins, which result in the highly regulated control of GPCR function.

G protein-coupled receptors (GPCRs)[1] represent the largest family of transmembrane signaling molecules in the human genome (*1*). As such, they interact with numerous intracellular molecules, which can act either to propagate or to curtail signaling from the receptor. Three critical cytoplasmic components that carry out these functions are the G proteins, G protein-coupled receptor kinases, and arrestins, which traditionally enable or inhibit agonist-mediated signaling (*2*, *3*). However, arrestins have recently been demonstrated to initiate their own signaling pathways in addition to desensitizing G protein-mediated signaling (*4*−*6*). Each of these proteins is thought to interact with GPCRs in distinct ways. Whereas G proteins interact predominantly with unphosphorylated, agonist-occupied receptors and G protein-coupled receptor kinases must interact with agonist-activated receptors in various states of phosphorylation, arrestins interact with agonist-activated receptors only once they possess a minimal level of phosphorylation. In our previous studies, complete phosphorylation of the *N*-formyl peptide receptor (FPR) has been shown to inhibit G protein binding to this receptor, whereas it simultaneously allows arrestin binding to occur (*7*−*9*). For some GPCRs, including the β_2-adrenergic receptor and partially phosphorylated FPR, G proteins are thought to continue to interact with phosphorylated receptors (*7*, *10*), whereas arrestins interact poorly with nonphosphorylated receptors but can block the interaction of G proteins with phosphorylated receptors (*10*−*13*).

The FPR is a prototypical G_i protein-coupled chemoattractant receptor that is responsible for the activation of leukocytes in response to bacterial infection (*14*−*17*).

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[1] Abbreviations: GPCR, G protein-coupled receptor; FPR, formyl peptide receptor; A, arrestin; G, G protein; GRKs, G protein-coupled receptor kinases; fMLFK−FITC, *N*-formyl-Met-Leu-Phe-Lys−fluorescein 5-isothiocyanate; fMLFF, *N*-formyl-Met-Leu-Phe-Phe.

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Following activation by ligand, the FPR has been shown to undergo phosphorylation within its carboxy terminus, a process that has been demonstrated to be required for both desensitization and receptor internalization (*18*−*20*). Our previous results have suggested that lower levels of phosphorylation are required for internalization than for desensitization (*21*). Furthermore, the ability to desensitize correlated with the ability to bind arrestin, as determined both in vivo and in vitro (*8*). An unexpected finding was that the ability of G proteins to bind to the FPR was inversely related to the ability of arrestins to bind to the FPR (*8*, *9*). Thus, partially phosphorylated forms of the FPR (with four potential phosphorylation sites present) continued to bind G proteins but did not bind arrestins and therefore did not functionally desensitize. Addition of two more potential phosphorylation sites reverted the properties of the FPR mutant to those of the wild-type receptor, namely, restoring the ability to bind arrestin and desensitize functionally. The unexpected result, however, was that G proteins were no longer capable of binding to these phosphorylated forms of the FPR in this reconstituted system, even in the absence of arrestins. These results raised the question as to whether, in vivo, phosphorylation of the FPR alone is sufficient to modulate the interaction of G proteins with the FPR.

To begin to address this question, we generated a fusion protein between the *N*-formyl peptide receptor and the $G_{\alpha i2}$ protein. Under these circumstances, the $G_{\alpha i2}$ protein would always be present at a high local concentration, not having the ability to diffuse away. Previous studies have shown that such constructs can be functional and in general lead to an increased coupling between the receptor and the linked G protein (*22*, *23*). A human β_2-adrenergic receptor−$G_{\alpha s}$ fusion protein expressed in ras-dependent murine carcinoma cell lines was capable of preventing tumor growth in syngeneic mice and inhibited cAMP-sensitive cancer cell proliferation (*24*, *25*). Fusion of the β_2-adrenergic receptor with the short form of $G_{\alpha s}$ results in a receptor resistant to homologous desensitization (*22*), while fusion of the β_2-adrenergic receptor with the long form of $G_{\alpha s}$ makes the receptor constitutively active, due to the lower GDP affinity of the $G_{\alpha s}$ variant (*26*). Quantitative analyses of receptor activation and the effects of point mutations in both receptor and G protein moieties on ligand efficacy have been demonstrated in chimeric α2A-adrenoceptor−$G_{\alpha i1}$ and A1 adenosine receptor−$G_{i/o}$ proteins (*27*−*30*). Chimeric FPR−G_{α} proteins have also been used to examine aspects of receptor−G protein coupling in membranes (*31*, *32*). In none of these studies were aspects of receptor processing, including desensitization, arrestin association, receptor internalization, and recycling, examined. In this study, we have used both cellular and noncellular approaches to test the functional capabilities of an FPR−$G_{\alpha i2}$ protein. Our results demonstrate that fusion of the $G_{\alpha i2}$ protein to the FPR does not prevent receptor desensitization or internalization. Furthermore, in vivo, arrestin is able to associate with the chimeric receptor following stimulation. However, in vitro, we find that the phosphorylated state, as well as the unphosphorylated state of the fusion protein, is capable of functionally binding the linked G protein. This suggests that, at sufficiently high local concentrations, G proteins can interact with phosphorylated GPCRs and raises the question as to whether, under physiological conditions in the cell, G proteins exist at sufficient concentrations in the vicinity of phosphorylated receptors for signaling to continue.

EXPERIMENTAL PROCEDURES

Reagents. All chemicals were from Sigma except where otherwise noted. Mixed bovine brain G proteins and *n*-dodecyl β-D-maltoside were from Calbiochem. Arrestin-2 and truncated arrestin-2 were purified as previously described (*33*). Anti-arrestin rabbit polyclonal antiserum was a generous gift from Dr. Jeffrey Benovic (Thomas Jefferson University). Anti-$G_{\alpha i2}$ mouse monoclonal antibody was purchased from Upstate Biotechnology. Goat anti-mouse antibody-coated polystyrene beads were from Bangs Laboratories. FITC-conjugated agonists were from Peninsula Laboratories. Anti-FITC antibody was prepared as described previously (*34*). fNle-Leu-Phe-Nle-Tyr-Lys−Alexa-633 was synthesized as follows. Alexa Fluor 633 carboxylic acid succinimidyl ester (Molecular Probes) and fNle-Leu-Phe-Nle-Tyr-Lys (Sigma) were each dissolved in anhydrous dimethyl sulfoxide to 2 mM. Equal volumes were incubated at room temperature overnight, and the product was used directly.

Generation of FPR−$G_{\alpha i2}$ Fusion Constructs. The human FPR (containing an *Eco*RI site and a *Not*I site embedded within the 5′ and 3′ primers, respectively) and rat $G_{\alpha i2}$ (containing a *Not*I site and an *Eco*RI site embedded within the 5′ and 3′ primers, respectively) were amplified by standard PCR protocols using Taq polymerase (Perkin-Elmer). The digested PCR products were ligated into *Eco*RI-digested and -phosphatased pSFFV.neo and screened for orientation of the insert. Appropriate clones were confirmed by dideoxy sequence analysis. The final fusion protein contained three alanine residues between the last amino acid of the FPR and the first amino acid of the G_{α} protein open reading frame.

Cell Culture and Transfection of U937 Cells. U937 cells were grown in tissue culture medium [RPMI (Hyclone) containing 10% FBS (Hyclone), 2 mM L-glutamine, 10 mM HEPES, 10 units/mL penicillin, 10 μg/mL streptomycin, and 0.001% ciprofloxin (Bayer)] at 37 °C in a humidified 5% CO_2 atmosphere. Cells were passaged from near confluent cultures every 3−4 days by reseeding at 2×10^5 cells/mL and expanded for membrane preparations to sealed, 5% CO_2 equilibrated, 1 L, baffled spinner flasks (Pyrex) and incubated at 37 °C. U937 cells (4×10^6) were transfected with DNA (1 μg) using Effectene as recommended by the manufacturer (Qiagen). For selection, G418 was added to a final active concentration of 1 mg/mL for 4−5 weeks.

Flow Cytometric Analysis of Cell Surface Receptors. Expression of the FPR and chimeric receptor in U937 cells was determined as follows. Cells (1×10^6) were harvested by centrifugation, washed once with PBS, and resuspended in 0.5 mL of cold PBS. The cells were then incubated with fNle-Leu-Phe-Nle-Tyr-Lys−FITC (10 nM) for at least 15 min on ice, followed by analysis on a FACSCalibur flow cytometer (Becton Dickinson) for fluorescent intensity. Dead cells and cell debris were excluded with a gate on forward and side scatter. Nonspecific binding was determined in the presence of 10 μM fMLF.

Ligand Binding Affinity. U937 cells expressing the wild-type FPR or fusion protein were washed and resuspended in 0.5 mL of cold PBS. Each sample contained 10000 cells

and was incubated on ice for at least 15 min with the indicated concentration of fluorescent agonist fNle-Leu-Phe-Nle-Tyr-Lys−FITC. Nonspecific binding samples were incubated with 10 μM nonfluorescent agonist fMLF for 5 min prior to the addition of fluorescent agonist. All samples were subjected to flow cytometric analysis on a FACSCalibur, and the mean channel fluorescence (MCF) was used to assess fluorescent agonist binding at equilibrium.

Receptor Internalization. Receptor internalization was carried out by incubating U937 cells expressing WT FPR and FPR−$G_{\alpha i2}$ fusion protein as well as the internalization-deficient FPR mutant ΔST with the agonist fMLF (1 μM) in TBS buffer at 37 °C for 0, 2, 4, 6, 8, and 10 min. At each time point, internalization was stopped by the addition of a large volume of cold TBS, and the samples were placed on ice. The cells were washed three times with cold TBS, and the receptors remaining on the cell surface were detected by incubation with 10 nM fNle-Leu-Phe-Nle-Tyr-Lys−FITC, followed by analysis on a FACSCalibur flow cytometer.

Receptor Recycling. The ability of receptors to recycle was determined by incubating U937 cells expressing either the WT FPR or FPR−$G_{\alpha i2}$ fusion protein with 1 μM fMLF in TBS buffer at 37 °C for 10 min to allow the receptors to internalize. The cells were then chilled by addition of an excess amount of cold buffer. Following three wash steps, cells were placed in a 37 °C water bath to allow receptor recycling to begin. At the indicated time, an aliquot of cells (0.5×10^6) was taken out and placed into an Eppendorf tube containing a large volume of prechilled TBS on ice. Cell surface receptors were detected by incubation with 10 nM fNle-Leu-Phe-Nle-Tyr-Lys−FITC for 15 min on ice, followed by FACSCalibur analysis. Maximum cell surface receptor expression was obtained by directly incubating untreated cells with 10 nM fNle-Leu-Phe-Nle-Tyr-Lys−FITC for 15 min on ice. Nonspecific binding was determined in the presence of 10 μM fMLF.

Cell Stimulation and Membrane Preparation. U937 cells expressing either WT FPR or FPR−$G_{\alpha i2}$ fusion protein were incubated with 10 μM fMLF in PBS for 8 min at 37 °C. Stimulation under these conditions results in greater than 90% maximal receptor phosphorylation (*19*). Immediately after stimulation, the tubes were placed on ice, and an equal volume of ice-cold PBS was added.

For membrane preparations, cells (either stimulated or not) were collected by centrifugation and resuspended at 10^7 cells/mL at 4 °C in cavitation buffer (10 mM HEPES, 100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 0.6 mg/mL ATP, and 1× protease inhibitor cocktail). The cells were disrupted by nitrogen cavitation at 500 psi for 15 min at 4 °C. Cell nuclei and debris were separated by two successive centrifugation steps at 1000*g* for 5 min at 4 °C. The supernatant, which contained the membranes, was pelleted by centrifugation at 135000*g* for 30 min and resuspended in HEPES−sucrose buffer (200 mM sucose, 25 mM HEPES, pH 7) in the presence of 1× protease inhibitor cocktail as well as phosphatase inhibitor cocktail for membranes from stimulated cells. Aliquots of membrane preparations were stored at −80 °C until use.

Receptor Solubilization with n-Dodecyl β-D-Maltoside. Membrane preparations were thawed, and 1.5 volumes of ice-cold HPSM buffer (30 mM HEPES, 100 mM KCl, 20 mM NaCl, 0.5 mM $MgCl_2$, pH 7.3) was added into the sample. Membranes were collected by centrifugation at 14000*g* for 30 min in an Eppendorf centrifuge at 4 °C. The supernatant was removed, and the pellet was resuspended in 300−900 μL of HPSM containing 1% *n*-dodecyl β-D-maltoside (DHPSM) and 1× protease inhibitor cocktail set 1. The suspension was gently mixed by passing it through a 1 mL syringe needle (26 gauge), followed by a 90 min incubation at 4 °C with gentle mixing. The insoluble fraction was separated by centrifugation at 14000*g* for 30 min in an Eppendorf centrifuge at 4 °C or at 70000*g* for 5 min in a Beckman airfuge. The supernatant, which contained the solubilized receptor proteins, was placed on ice and used immediately for experimentation.

Sucrose Gradient Velocity Sedimentation. Sucrose solutions (5%, 10%, 15%, and 20% sucrose) were made in HPSM buffer containing 1% *n*-dodecyl β-D-maltoside and 1× protease inhibitor cocktail set 1. Linear sucrose density gradients were prepared by placing 155 μL of each sucrose solution sequentially into a 5 × 41 mm Ultra-Clear centrifuge tube (Beckman). GTPγS (10 μM) was included in the gradients for samples treated with GTPγS. The tubes were equilibrated for 6−8 h at room temperature to allow for the formation of linear gradients. Solubilized WT FPR or FPR−$G_{\alpha i2}$ fusion protein membrane preparations (25 μL containing 1.67×10^7 cell equivalents), with or without a 45 min preincubation with 1 mM GTPγS, were layered on top of the sucrose gradients. The gradients were centrifuged in a SW 55 Beckman swinging-bucket rotor for 16 h at 35000 rpm (148862g_{max}) at 4 °C and subsequently manually fractionated into 13 × 50 μL fractions. Each fraction was incubated with 5 nM fMet-Leu-Phe-Lys−FITC for 90 min on ice. The majority of each fraction (40 μL) was diluted to 200 μL with DHPSM and subjected to spectrofluorometric analysis to access the receptor distribution in the gradients. A mixture of 5 μg of bovine serum albumin (4.4 S) and rabbit immunoglobulin G (7.7 S) was used as protein standards and centrifuged in parallel with the experimental gradients. These fractions were analyzed by SDS−polyacrylamide gel electrophoresis, followed by Coomassie Blue staining. On the basis of the sedimentation rate of these standard proteins, the 4S and 7S proteins were calculated to peak in fractions 6 and 8, respectively.

Immunodetection of FPR−$G_{\alpha i2}$ Fusion Protein. Polystyrene beads covalently coated with goat anti-mouse antibodies (GAM beads; Bangs Laboratories) were further coated with an anti-$G_{\alpha i2}$ mouse monoclonal antibody as follows. GAM beads (25 μL, 1×10^8 beads/mL) were washed and resuspended in HPSM buffer and incubated with 2 μg of anti-$G_{\alpha i2}$ Ab or anti-hexahistidine Ab (serving as an Ig isotype control) in 100 μL for 2 h at 4 °C with mixing. The beads were then collected by centrifugation, washed twice with HPSMD buffer (HPSM buffer with 0.1% *n*-dodecyl β-D-maltoside), and resuspended in 100 μL of HPSMD. Solubilized membrane preparations of WT FPR and FPR−$G_{\alpha i2}$ fusion protein (containing approximately 65 nM receptor) were incubated with 2 μL of $G_{\alpha i2}$-coated GAM beads (about 5×10^4 beads) and 700 nM fluorescent agonist fMLFK−FITC. Incubations were carried out in either the absence or presence of GTPγS (3 μM) in a final reaction mixture of 10 μL in a 96 V well plate (Costar) at 4 °C for 90 min with shaking. The volume of each sample was brought to 200 μL with HPSMD, and the fluorescence

intensity was analyzed on a FACScan flow cytometer. Debris and bead doublets were excluded with a gate on forward and side scatter. Incubation of the membrane preparations with GTPγS prior to solubilization followed by a wash step resulted in virtually the same results as the direct addition of GTPγS to the reaction mixture.

Ca^{2+} Mobilization. Ca^{2+} mobilization assays were performed as previously described (*35*, *36*). U937 cells expressing WT FPR or fusion protein were harvested by centrifugation, washed, and resuspended in HBSS buffer containing Ca^{2+}. The cells were incubated with 5 μM indo-1AM (Molecular Probes) for 30 min at 37 °C with rotation, followed by centrifugation and an additional wash with HBSS. The cells were finally resuspended in HBSS at 15×10^6 cells/mL and kept on ice. Intracellular calcium mobilization of approximately 0.5×10^6 cells following stimulation with fMLF was monitored by continuous fluorescent measurement at 405 and 480 nm on a SLM 8000 spectrofluorometer using photon counting mode.

Receptor Desensitization. U937 cells were loaded with indo-1AM as described above. Each sample was then split into two aliquots. Cells were incubated in the presence or absence of 500 nM fMLF at 37 °C for 8 min, followed by two washes with HBSS containing Ca^{2+} at room temperature. For each wash, the cells were pelleted by a 30 s centrifugation in an Eppendorf centrifuge. The total time for washing was approximately 4 min. Cells in each sample were resuspended in HBSS at 15×10^6 cells/mL and placed on ice. Intracellular calcium mobilization was monitored as described above.

Confocal Microscopy. U937 cells were harvested, washed, and resuspended in PBS. The cells were incubated with 10 nM fNle-Leu-Phe-Nle-Tyr-Lys−Alexa-633 and incubated at 37 °C for 8 min or at 0 °C (control). Following a wash with cold PBS and fixing with 2% paraformaldehyde (30 min on ice), the cells were permeabilized with 0.02% saponin. Permeabilized cells were incubated with an anti-arrestin-2 polycolonal rabbit antiserum and a mouse anti-$G_{\alpha i2}$ antibody for 30 min at room temperature. Following a wash, cells were incubated with a mixture of a Texas Red-conjugated goat anti-rabbit secondary antibody and a FITC-conjugated goat anti-mouse secondary antibody for another 30 min at room temperature. After three washes, the cells were resuspended in Vectashield (Vector Laboratories) and mounted on slides. Fluorescence images were acquired on a Zeiss LSM 510 confocal microscope.

Reconstitution of FPR with G Proteins or Arrestin-2. Membrane preparations were solubilized in 1% DOM as described above. The solubilized receptors were incubated in a final volume of 10 μL with various concentrations of bovine brain G proteins, arrestin-2, truncated arrestin-2 (1−382), or control buffer in the presence of 20 nM fluorescent ligand, fMLFK−FITC. Blocked samples were incubated with the nonfluorescent ligand fMLFF (20 μM) 5 min prior to the addition of fluorescent ligand. GTPγS (2 mM final) was added to samples to block G protein association. The samples were incubated on ice for 90 min in the dark and then subjected to spectrofluorometric analysis.

Spectrofluorometric Analysis. Analysis of reconstituted samples was performed as described before (*9*, *37*). Briefly, following the reconstitution of receptors with G proteins or arrestin-2 in the presence of fluorescent ligand, the sample volume was brought to 200 μL with HPSMD containing protease inhibitor cocktail 1. The fluorescence associated with the ligand fMLFK−FITC was measured with an SLM 8000 spectrofluorometer at 22 °C with constant stirring. During real time kinetic studies, the total fluorescence from free and receptor-bound ligands was monitored for 10 s, followed by addition of a high-affinity anti-FITC antibody into the sample. Fluorescence contributed by the unbound, FITC-conjugated ligand was immediately quenched by the antibody. Therefore, the remaining fluorescence represented the receptor-bound ligand. At 40 s into the assay, 100 μM (final) GTPγS was added to evaluate the coupling of FPR with G proteins and arrestins based on the characteristic ligand dissociation rates. Data were acquired for 100 s with a 0.5 s integration. The detergent concentration was maintained above the critical micelle concentration throughout the experiment.

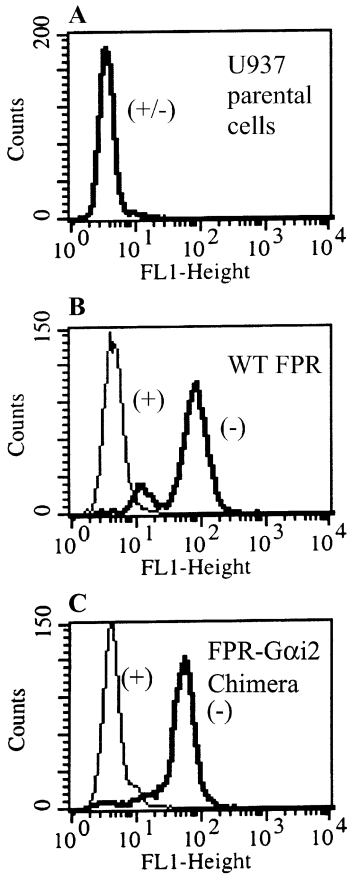

FIGURE 1: Analysis of FPR−$G_{\alpha i2}$ chimeric protein expression in U937 cells by flow cytometry. Cell surface FPR expression of untransfected U937 cells (A), stably transfected WT FPR U937 cells (B), and stably transfected FPR−$G_{\alpha i2}$ U937 cells was determined. Fluorescent ligand (10 nM fnLLFnLYK−FITC) was incubated with cells on ice in the absence (−) or presence (+) of 10 μM fMLF. Histograms are representative of three experiments with duplicate samples in each experiment.

RESULTS

Analysis of Ligand Binding to Chimeric FPR−$G_{\alpha i2}$ in U937 Cells. The chimeric FPR−$G_{\alpha i2}$ protein was generated by PCR amplification of the FPR and $G_{\alpha i2}$ open reading frames containing in-frame *Not*I restriction sites at the 3′ end of the FPR and 5′ end of the $G_{\alpha i2}$ protein. The final

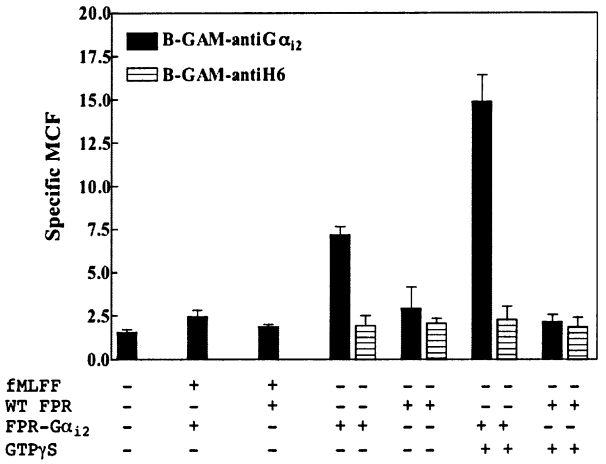

FIGURE 2: Detection of solubilized chimeric FPR−$G_{\alpha i2}$ protein. Goat anti-mouse Ab-coated polystyrene beads (50000) were incubated with mouse monoclonal anti-$G_{\alpha i2}$ Abs (solid bars) or anti-H6 Abs as an Ig isotype control (shaded bars), as described in Experimental Procedures. Detergent-solubilized FPR−$G_{\alpha i2}$ and WT FPR (65 nM), with or without preincubation with 1 mM GTPγS, were mixed with the coated beads. Fluorescent ligand fMLFK−FITC (70 nM) was added in a 10 μL reaction and mixed at 4 °C for 90 min. In control-blocked samples, 10 μM nonfluorescent agonist fMLFFGGK was added into the reaction mixture 5 min prior to the addition of fluorescent ligand. Individual samples were diluted to 200 μL and subjected to flow cytometric analysis. Data shown are the average of two experiments with duplicate samples in each.

construct, coding for three alanines between the last amino acid of the FPR and the first amino acid of the $G_{\alpha i2}$ protein, was stably transfected into U937 cells. Cell surface expression of the FPR−$G_{\alpha i2}$ chimeric protein in U937 cells was determined by flow cytometric analysis using a fluorescinated ligand (Figure 1). A previously characterized U937 cell line expressing wild-type FPR was used as a positive control and the untransfected U937 cells as a negative control. While the nontransfected U937 cells showed no specific binding to the fluorescinated ligand, addition of fMLF blocked the binding of fMLFK−FITC to U937 cells transfected with either the WT FPR or the chimeric FPR−$G_{\alpha i2}$ protein (Figure 1).

Detection of the Chimeric FPR−$G_{\alpha i2}$ Protein. To differentiate between the WT FPR and the chimeric FPR−$G_{\alpha i2}$ protein, we developed a novel bead-based flow cytometry immunoassay, using polystyrene beads coated with goat anti-mouse antibodies (GAM beads), an anti-$G_{\alpha i2}$ mouse monoclonal antibody, and detergent-solubilized receptors. As seen in Figure 2, specific binding of the fluorescent ligand was detected only when the chimeric protein was bound to the anti-$G_{\alpha i2}$ antibody. Controls showed that binding was dependent upon the specific antibody and unique to the chimeric protein with little binding of the wild-type receptor. The binding was increased by GTPγS, suggesting that activation of the fusion protein and dissociation of the $\beta\gamma$ subunits enhanced access to the monoclonal antibody.

To further evaluate whether the G protein $\beta\gamma$ subunits were associated with the solubilized chimeric FPR−$G_{\alpha i2}$ protein, we utilized sucrose gradient velocity sedimentation. The FPR sediments as a 4S species, reconstitutes with G protein as a 7S species, and returns to 4S with the addition of GTPγS (*36*−*39*). We compared the sedimentation profiles of the solubilized chimeric FPR−$G_{\alpha i2}$ protein and that of the WT

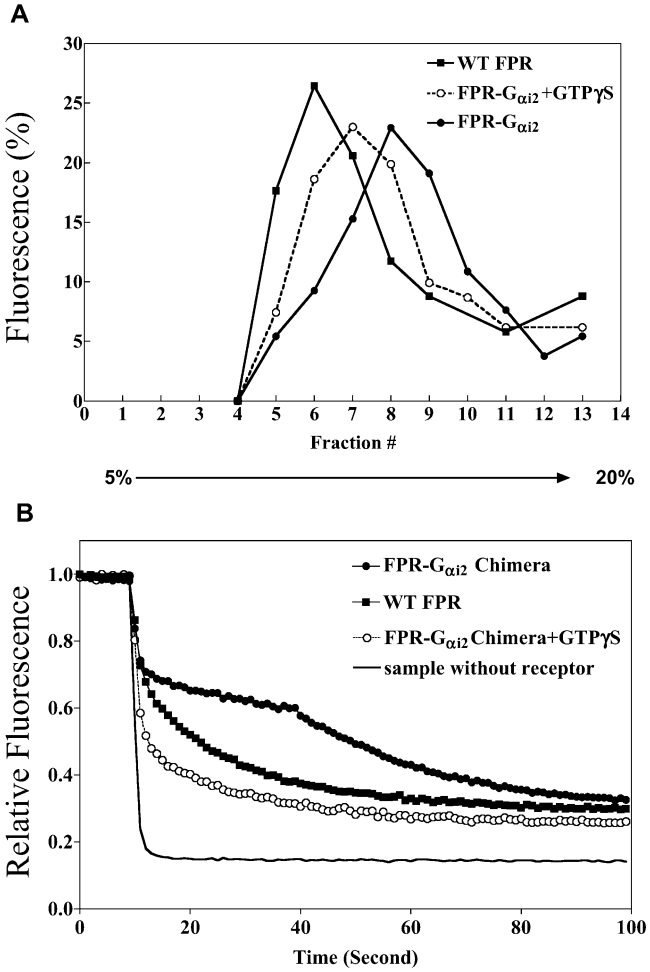

FIGURE 3: Sedimentation profiles of the FPR and the chimeric FPR−$G_{\alpha i2}$ protein by sucrose gradient sedimentation analysis. Membrane preparations of the WT FPR and the chimeric FPR−$G_{\alpha i2}$ were solubilized and subjected to sucrose gradient sedimentation as described in Experimental Procedures. Gradients were then manually fractionated, and each fraction was incubated with 5 nM fMLFK−FITC for 90 min on ice for spectrofluorometric analysis of receptor content. Sedimentation profiles shown in panel A are WT FPR (■), FPR−$G_{\alpha i2}$ preincubated with GTPγS (○), and FPR−$G_{\alpha i2}$ (●). Panel B is the spectrofluorometric real time analysis of peak fractions from each sample. Data represent a single determination of three independent experiments.

FPR in 5−20% sucrose gradients (Figure 3A). The presence of the FPR in each fraction was determined by fluorescent ligand binding to the receptor following quenching of free ligand (*37*). Whereas the WT FPR sedimented as a 4S species with low ligand affinity (Figure 3B), the chimeric FPR−$G_{\alpha i2}$ protein sedimented as a 7S species, identical to that previously observed for the WT FPR reconstituted with exogenous G proteins (*36*). Furthermore, fluorometric analysis of the ligand dissociation rate from the peak samples demonstrated that the ligand binding of the 7S species was characteristic of a high-affinity, GTPγS-sensitive G protein complex (Figure 3B). To assess whether the chimeric FPR−$G_{\alpha i2}$ protein was likely to contain bound G protein $\beta\gamma$ subunits, we treated the chimeric FPR−$G_{\alpha i2}$ protein with GTPγS and analyzed the sedimentation profile (Figure 3A). The resulting profile yielded a peak midway between the 4S FPR and 7S chimeric FPR−$G_{\alpha i2}$ protein, again with low ligand affinity (Figure 3B). This shift would be consistent with a reduction in mass equivalent to the loss of the $\beta\gamma$

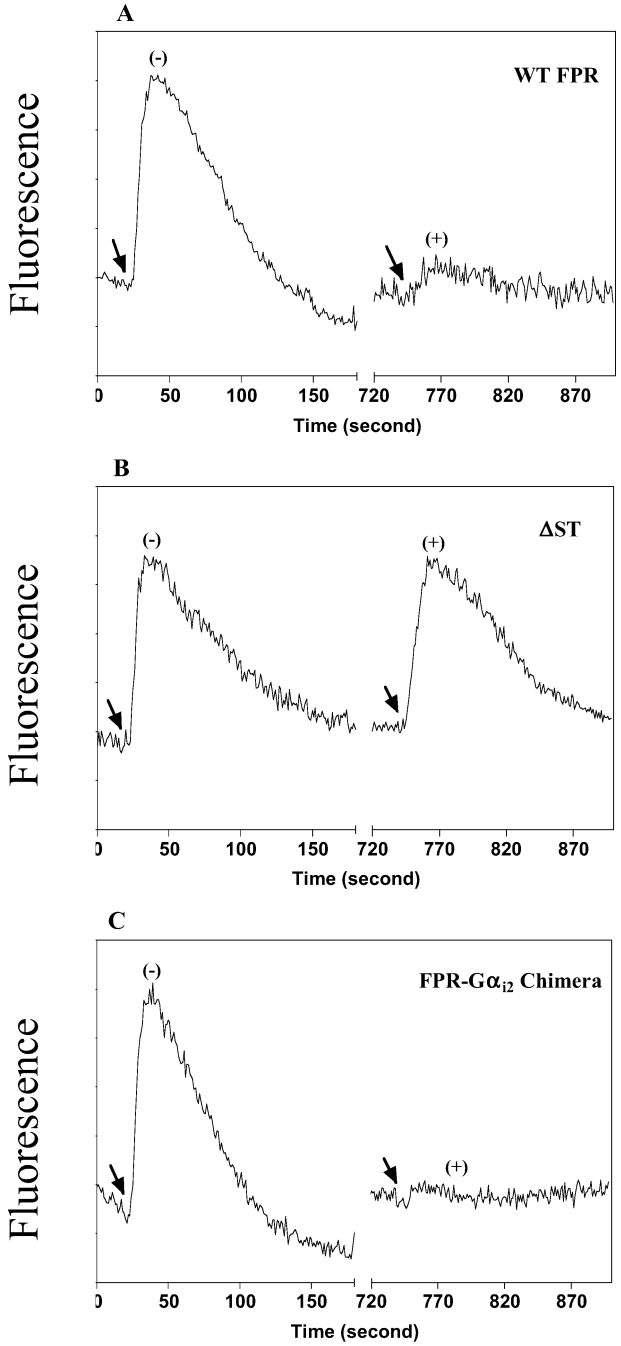

FIGURE 4: Receptor desensitization following stimulation. U937 cells expressing WT FPR (panel A), a desensitization-deficient mutant FPR, ΔST (panel B), or a chimeric FPR−$G_{\alpha i2}$ protein (panel C) were loaded with indo-1AM. Each sample was then split into two aliquots. One was treated with 500 nM fMLF at 37 °C for 8 min (+), while the other was not (−). Stimulation was followed by two washes with HBSS containing Ca^{2+} at room temperature. Prestimulated and unstimulated cells were then exposed to 500 nM fMLF, and the elevation of intracellular calcium was monitored. Data are a representative of three independent experiments.

subunits, suggesting that the solubilized, chimeric FPR−$G_{\alpha i2}$ protein originally contained the $\beta\gamma$ subunits.

Chimeric FPR−$G_{\alpha i2}$ Protein Function in U937 Cells. To examine the functions of both the FPR and the $G_{\alpha i2}$ moieties of the chimeric protein in vivo, a series of cell-based assays were conducted. First, we compared the ligand binding ability of U937 cells transfected with WT FPR or chimeric FPR−$G_{\alpha i2}$ protein. U937 cells transfected with WT FPR and FPR−$G_{\alpha i2}$ protein displayed similar single exponential ligand

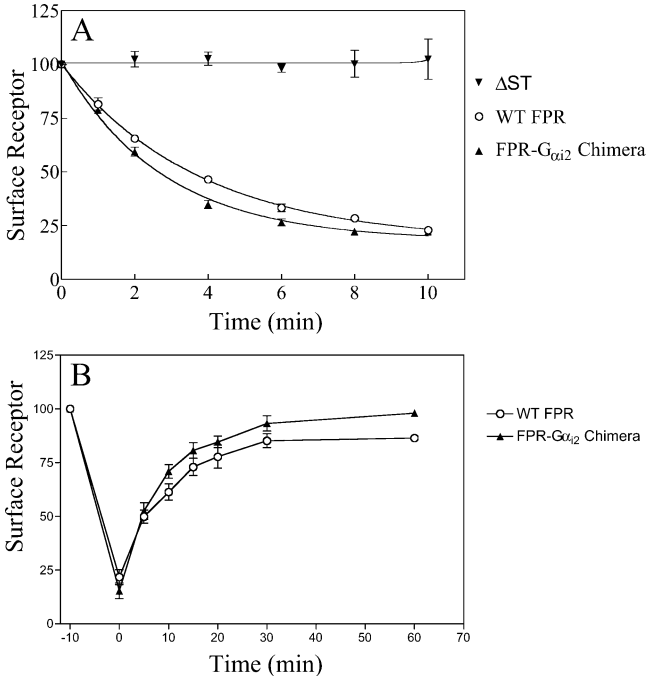

FIGURE 5: Receptor internalization and recycling. (A) U937 cells expressing the WT FPR (○), the chimeric receptor protein (▲), or the internalization-deficient mutant ΔST (▼) were washed and incubated with 1 μM agonist fMLF at 37 °C for various times. The cells were washed with cold TBS, and the receptors remaining on the cell surface were detected by incubation with 10 nM fluoresceinated agonist fMLFK−FITC on ice, followed by flow cytometric analysis. (B) WT FPR (○) and chimeric FPR−$G_{\alpha i2}$ protein (▲) expressing U937 cells were incubated with 1 μM fMLF at 37 °C for 10 min to induce receptor internalization. The samples were then washed three times and warmed to 37 °C to allow receptor recycling to the cell surface. At the indicated times, an aliquot from each sample was placed on ice. Cell surface receptors were then detected by incubating the cells with 10 nM fnLLFnLYK−FITC for 15 min on ice and analyzed by flow cytometry. Maximum cell surface receptor expression was obtained by directly incubating the cells with fluorescent agonist. Nonspecific binding was determined by adding 500 μM fMLF to cells 5 min prior to the addition of fluorescent agonist. Data represent the mean ± SD of three to five experiments.

binding curves. The K_d for the chimeric protein transfected cells was 3.9 ± 0.8, and the K_d for WT FPR transfected cells was 4.3 ± 0.9 (data not shown). This result demonstrated that the FPR moiety of the chimeric protein displays a ligand binding ability similar to that of the nonfused FPR (*22*, *31*).

To test the signal transducing ability of the chimeric protein, intracellular calcium mobilization was measured upon ligand activation. We observed similar dose-dependent increases of intracellular calcium levels in U937 cells expressing either the WT FPR or the chimeric FPR−$G_{\alpha i2}$ protein. Calcium fluxes were similar at fMLF concentrations between 0.1 nM and 10 μM. The EC_{50} for WT FPR was 2.4 × 10^{-8} ± 0.1 and the EC_{50} for FPR−$G_{\alpha i2}$ protein was 2.8 × 10^{-8} ± 0.2 (data not shown). Furthermore, the calcium fluxes generated by the WT FPR and FPR−$G_{\alpha i2}$ fusion protein were sensitive to pertussis toxin pretreatment (data not shown). These results indicate that the fusion of the $G_{\alpha i2}$ protein to the FPR carboxy terminus does not interfere with the ability of the FPR to initiate signal transduction.

Desensitization to chronic or repeated activation is a fundamental property of GPCRs. To determine whether

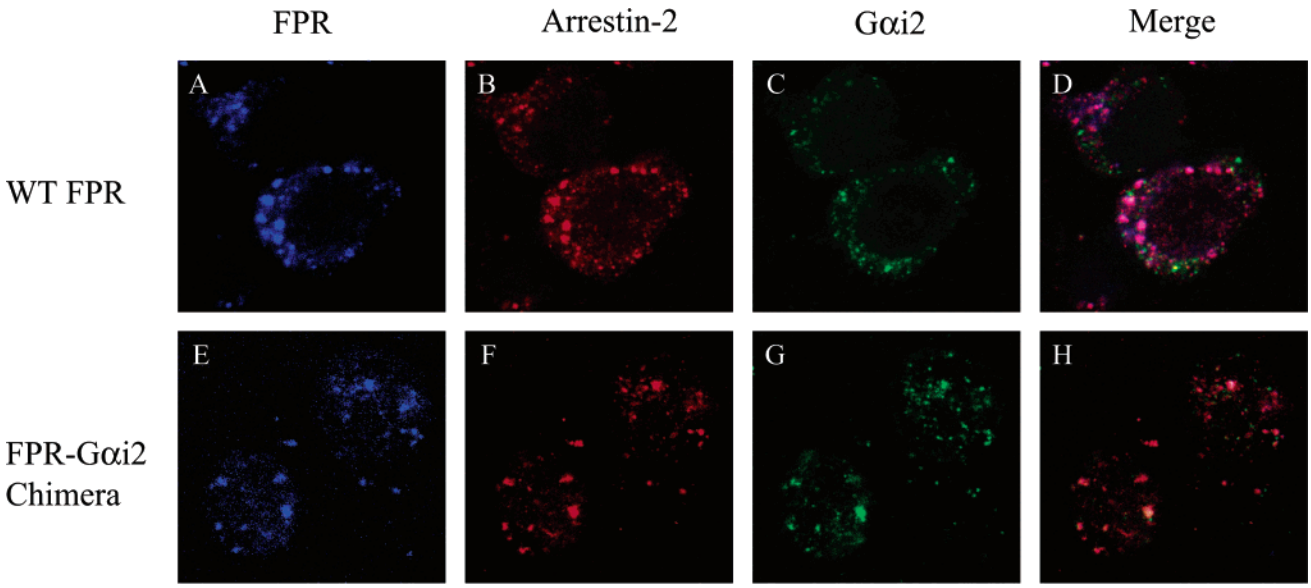

FIGURE 6: Colocalization of $G_{\alpha i2}$ protein with FPR and arrestin-2 in cells expressing chimeric FPR−$G_{\alpha i2}$ protein but not WT FPR. U937 cells expressing either the WT FPR or the chimeric FPR−$G_{\alpha i2}$ protein were incubated with 15 nM fNleLFNleYK−Alexa-633 (blue) at 37 °C for 8 min. After fixation and permeabilization, the cells were immunostained with primary Abs specific for arrestin-2 and $G_{\alpha i2}$, followed by incubation with Texas Red conjugated secondary Ab to detect arrestin-2 (red) and FITC-conjugated secondary Ab to detect $G_{\alpha i2}$ (green). Confocal images were taken with a Zeiss 510 LSM microscope and are representative of the majority of cells from two separate experiments.

functional desensitization occurred with the chimeric protein, we compared intracellular calcium mobilization in U937 cells expressing WT FPR, chimeric FPR−$G_{\alpha i2}$ protein or a nondesensitizing FPR mutant, ΔST (*8*, *21*). In response to an initial stimulation with 500 nM fMLF, U937 cells expressing WT FPR, chimeric FPR−$G_{\alpha i2}$ protein, and ΔST had similar intracellular calcium mobilization responses (Figure 4). When restimulated after removing fMLF, the wild-type FPR desensitizes (Figure 4, panel A) and the ΔST mutant does not (Figure 4, panel B). The FPR−$G_{\alpha i2}$ chimeric protein behaves like the WT FPR (Figure 4, panel C), suggesting that the FPR−$G_{\alpha i2}$ chimeric protein undergoes normal GRK-mediated phosphorylation and possibly binding of arrestin.

Chimeric FPR−$G_{\alpha i2}$ Protein Trafficking in U937 Cells. Using the internalization-deficient mutant ΔST as a negative control and WT FPR as a positive control, we measured the rate of internalization of the chimeric FPR−$G_{\alpha i2}$ protein. Internalization of the chimeric receptor expressed in U937 cells was similar in extent to that of WT FPR expressed in U937 cells (Figure 5A), with approximately 75% of cell surface receptors being internalized within 10 min of ligand addition. The half-time for internalization of the FPR−$G_{\alpha i2}$ chimeric protein (1.9 min) was similar to that of the WT FPR (2.6 min). The WT FPR and the FPR−$G_{\alpha i2}$ chimeric protein also displayed similar receptor recycling rates and magnitudes (Figure 5B); the half-times for recycling to the cell surface were 6.9 min for the WT FPR and 6.2 min for the chimeric protein. The results suggest comparable processes of receptor endocytosis, ligand removal, and receptor dephosphorylation.

To probe for arrestin interactions in the processing of the fusion protein, we compared the cellular localization of arrestins and G proteins following stimulation with Alexa-633-labeled ligand by confocal immunofluorescence microscopy. In unstimulated cells expressing either the WT FPR or the FPR−$G_{\alpha i2}$ chimeric protein, the receptor was expressed on the cell surface, the $G_{\alpha i2}$ protein appeared punctate and membrane-localized, and the arrestin-2 was diffusely distributed in the cytoplasm (data not shown). In cells expressing the WT FPR, arrestins but not G proteins were seen to colocalize with the liganded receptor following stimulation (Figure 6, panels A−D). The interactions with G proteins are expected to be transient and therefore not readily detected. In contrast, with the FPR−$G_{\alpha i2}$ chimeric protein, punctate clusters are formed upon internalization, which colocalized with both arrestins and a fraction of the $G_{\alpha i2}$ protein in the cells (Figure 6, panels E−H). The binding of arrestin to the FPR−$G_{\alpha i2}$ chimeric protein in vivo demonstrates that the chimeric receptor protein undergoes phosphorylation to levels comparable to that of the wild-type FPR, since we have previously shown that low levels of FPR phosphorylation are insufficient to permit arrestin binding both in vivo and in vitro (*8*). Substantial amounts of $G_{\alpha i2}$ did not colocalize with either the FPR or arrestin-2 (panels G and H), representing the native $G_{\alpha i2}$ protein, which is not fused to FPR.

Reconstitution of the Solubilized WT FPR and FPR−$G_{\alpha i2}$ Chimeric Protein with G Proteins and Arrestins. We have recently described the soluble reconstitution of the FPR with G proteins and arrestins (*8*, *9*, *37*) in which the unphosphorylated FPR binds exogenous G proteins in a high ligand affinity complex that is sensitive to GTPγS, whereas the fully phosphorylated FPR binds arrestin in a high ligand affinity complex that is insensitive to GTPγS. Since the phosphorylated WT FPR did not bind G proteins even in the absence of arrestins, we considered whether G proteins, at high local concentrations, might be capable of interacting with the phosphorylated FPR. To evaluate the interactions of the chimeric FPR−$G_{\alpha i2}$ protein with exogenous G proteins and arrestins, we extended our in vitro reconstitution assays using detergent-solubilized receptors (Figures 7 and 8).

Figure 7 shows the reconstitution of the WT FPR and the FPR−$G_{\alpha i2}$ chimeric protein with different concentrations of exogenous bovine brain G proteins (a mixture of $G_{\alpha i/o}$ subunits) in the presence of fluoresceinated ligand. Whereas

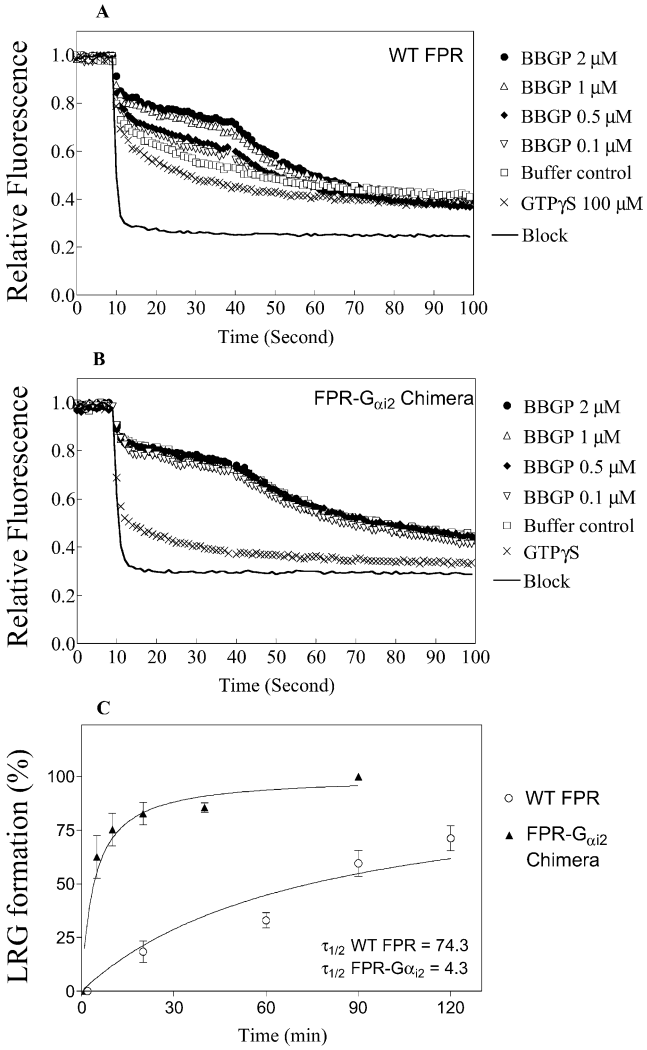

FIGURE 7: Reconstitution of solubilized WT FPR and chimeric FPR−$G_{\alpha i2}$ proteins with G proteins. Unstimulated membrane preparations from the WT FPR (panel A) and the chimeric FPR−$G_{\alpha i2}$ protein (panel B) were solubilized with 1% DOM as described in Experimental Procedures. The solubilized receptors were incubated with various concentrations of bovine brain G proteins (BBGP) or control buffer and 20 nM fluorescent agonist fMLFK−FITC for 90 min on ice. For blocked samples, 20 μM fMLFF was added prior to the fluoresceinated agonist. To uncouple the receptor from G proteins, GTPγS (100 μM) was added during the 90 min incubation. The receptor concentrations were estimated at 130 nM for the WT FPR and 25 nM for the chimeric receptor protein. For each sample, anti-FITC Ab was added at 10 s and GTPγS (100 μM) was added at 40 s. Panel C shows the kinetics of G protein reconstitution with WT FPR (○) and the chimeric FPR−$G_{\alpha i2}$ protein with no added G proteins (▲). Data points were expressed as percent of the maximum LRG reconstitution of chimeric FPR−$G_{\alpha i2}$ protein at 90 min. Data are a representative of three to four independent experiments with duplicate measurements in each.

the WT FPR required a high concentration of G proteins and long times of reconstitution (panels A and C), the fusion protein required no additional G proteins and only short times for reconstitution. The half-time for solubilized WT FPR reconstitute with G proteins in solution was 74 min, whereas that for the FPR−$G_{\alpha i2}$ chimeric protein was only 4 min (panels B and C). These results indicate that the high local concentration permits extensive and rapid interaction with the FPR.

The question therefore arises: do these high local G protein concentrations reverse the inhibitory effects of receptor phosphorylation that we have observed on G protein interactions and, if so, serve to compete with arrestin binding? Figure 8 shows the reconstitution of the phosphorylated chimeric and WT receptors with arrestin. Both WT arrestin-2 and truncated arrestin-2 increased the affinity of the phosphorylated WT FPR (Figure 8A), as we have previously shown (*9*). The high ligand affinity states of these receptor−arrestin complexes were not sensitive to GTPγS addition. As with the phosphorylated WT FPR, addition of the same concentrations of truncated arrestin-2 to the chimeric FPR−$G_{\alpha i2}$ protein also caused a shift in the ligand binding properties of the receptor, but to a lesser extent (Figure 8B). Addition of WT arr-2 resulted in little change in ligand binding to the chimeric FPR−$G_{\alpha i2}$ protein. However, in both cases, the high ligand affinity complex of the phosphorylated chimeric protein displayed GTPγS sensitivity, similar to that observed in the absence of added arrestins. To test whether the decrease in arrestin binding to the chimeric FPR−$G_{\alpha i2}$ protein was the result of competition of the fused $G_{\alpha i2}$ protein, assays were incubated in the presence of GTPγS during the reconstitution phase of the experiment. Putative activation of the $G_{\alpha i2}$ protein could eliminate a high-affinity G protein complex with the receptor. Under these circumstances, both the WT arr-2 and to a greater extent the truncated arr-2 induced a ligand affinity shift that was insensitive to subsequent addition of GTPγS (Figure 8C,D). These results confirm that the stimulated FPR−$G_{\alpha i2}$ chimeric protein is indeed phosphorylated and can bind the fused $G_{\alpha i2}$ protein as well as exogenous arrestin if the fused $G_{\alpha i2}$ protein is prevented from competing for binding to the phosphorylated receptor.

DISCUSSION

The interactions of GPCRs and their associated proteins are known to be complex and highly regulated. In addition, at each step in the activation and regulation of a GPCR, the receptor has alternate proteins with which it can interact. In the case of the ligand-bound but otherwise naive receptor, it can interact with G proteins and G protein-coupled receptor kinases. In this state, the receptor should be unable to interact effectively with arrestins. Once phosphorylated by a GRK, a process that enables desensitization and internalization, the receptor might be expected to demonstrate a decrease in G protein binding affinity as well as an increased affinity for internalization and desensitization machinery, such as arrestins (*11*). Very little is known, however, of the relative affinities of these accessory proteins, either in vitro or in vivo, for GPCRs in various states of phosphorylation. Our recent results have suggested that the fully phosphorylated FPR is deficient in its ability to bind G proteins, in contrast to the prevailing paradigms (*7*−*9*, *37*). To test the effects of increased local concentrations of G proteins on their ability to interact with phosphorylated forms of the FPR, we generated and characterized a chimeric FPR−$G_{\alpha i2}$ protein. Although the ability of such chimeric proteins to couple functionally and undergo activation has been previously demonstrated (*22*, *23*, *28*, *31*, *32*, *40*), little is known about the subunit composition, desensitization, internalization, or molecular assemblies of such precomplexed proteins.

In this study, we have demonstrated that the chimeric FPR−$G_{\alpha i2}$ protein exhibits appropriate ligand affinity as well as signaling capacities similar to those of the WT FPR. Our

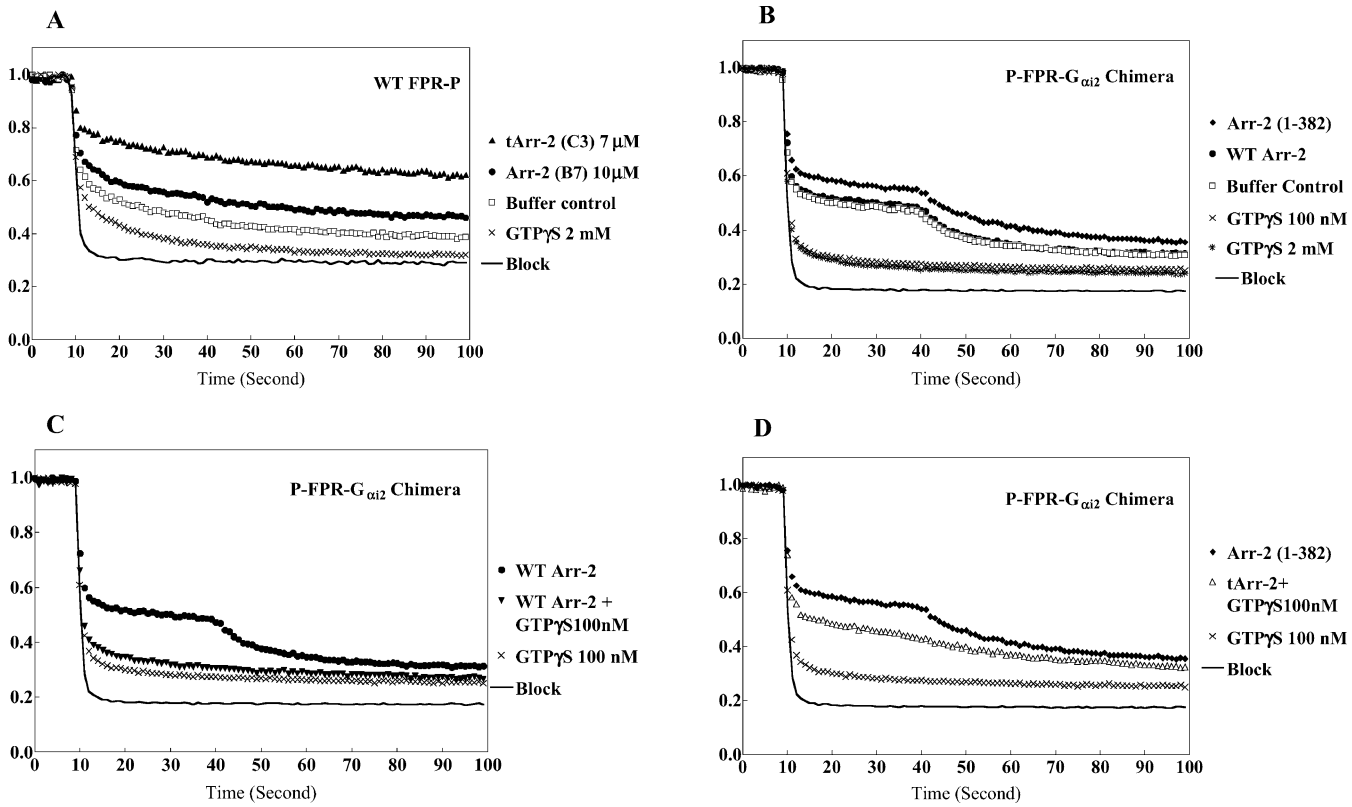

FIGURE 8: Reconstitution of solubilized receptors with arrestins. Membrane preparations from agonist-stimulated WT FPR transfected cells (panel A) and stimulated chimeric FPR−$G_{\alpha i2}$ protein transfected cells (panels B−D) were solubilized with 1% DOM. The solubilized receptors were incubated with either 10 μM WT arrestin-2, 7 μM truncated arrestin-2 (1−382), 100 nM GTPγS or control buffer as indicated, and 20 nM fluorescent agonist fMLFK−FITC for 90 min on ice. The receptor concentrations were estimated at 78 nM for phosphorylated WT FPR and 14 nM for phosphorylated chimeric protein. Blocked samples were incubated with 20 μM fMLFF prior to the addition of fluorescent ligand. For each sample, anti-FITC Ab was added at 10 s, and GTPγS (100 μM) was added at 40 s. Data are a representative of three independent experiments with duplicate measurements in each.

results also indicated that, when solubilized, the chimeric receptor contained G protein $\beta\gamma$ subunits, which could be dissociated by treatment with GTPγS. This is consistent with the ability of the chimeric FPR−$G_{\alpha i2}$ protein to stimulate intracellular calcium mobilization in response to ligand, a process initiated by dissociated $\beta\gamma$ subunits. Surprisingly, the processing events typically observed with the FPR were little perturbed by the carboxy-terminal attachment of the G protein αi2 subunit. In fact, internalization and recycling of the chimeric FPR−$G_{\alpha i2}$ protein were very similar to those of the wild-type FPR. This suggested that the presence of the G protein αi2 subunit did not interfere with phosphorylation of the FPR's carboxy terminus by GRKs. This conclusion is further supported by the observation that the chimeric FPR−$G_{\alpha i2}$ protein desensitized to an extent similar to that of the WT FPR. Since we have previously shown that G proteins can still interact with partially phosphorylated forms of the FPR, yet arrestins require almost complete FPR phosphorylation, the level of receptor phosphorylation is likely similar to that of the WT receptor. It further demonstrates that receptor processing (phosphorylation or its sequelae) is able to prevent G protein coupling in vivo even with a high local concentration of G proteins. One of the consequences of receptor phosphorylation is arrestin binding. Confocal fluorescence microscopy revealed that arrestins colocalized with internalized fluorescent agonist in endosomes of wild-type FPR and chimeric receptor-expressing cells. Furthermore, in the case of the chimeric receptor, this occurred despite the presence of the G protein αi2 subunit, which confirmed the presence of the chimeric FPR−$G_{\alpha i2}$ protein in endosomes with the fluorescent ligand and arrestin. G protein αi2 subunits were not seen to colocalize with the WT FPR, suggesting that, under physiological conditions, G proteins do not traffic with the FPR into endosomes. Such a segregation of FPR and G proteins has been previously described with respect to light and heavy membrane fractions (*41*). Last, analysis of the recycling rate of the receptors demonstrated that the chimeric FPR−$G_{\alpha i2}$ protein trafficked back to the cell surface with kinetics similar to that of the WT FPR, suggesting that the presence of the G protein αi2 subunit did not interfere with plasma membrane localization signals.

Together, these results indicate that the chimeric FPR−$G_{\alpha i2}$ protein is able to function in a very similar way in vivo to the WT FPR, undergoing appropriate activation, desensitization, internalization, and recycling. To further examine the effects of the high local concentrations of G protein αi2 subunits in receptor desensitization, we also reconstituted the solubilized form of the chimeric FPR−$G_{\alpha i2}$ protein to examine the molecular interactions in the absence and presence of arrestins. Such studies cannot be carried out in vivo due to the universal presence of nonvisual arrestins in all cell types (*42*). Incubation of the solubilized, chimeric FPR−$G_{\alpha i2}$ protein with fluorescent ligand resulted in the very rapid formation of a ternary ligand−receptor−G protein complex with high affinity for ligand that was also sensitive to the addition of GTPγS. To attain a similar complex with the WT FPR required the addition of 1−2 μM exogenous G

protein and incubation times of 60−90 min as compared to 5−10 min for the chimeric FPR−$G_{\alpha i2}$ protein with no exogenous G protein added. In fact, further addition of exogenous G proteins did not increase the formation of the high ligand affinity complex, suggesting that the maximal level of ternary complex formation had been achieved with the chimeric receptor.

Analysis of the phosphorylated, solubilized, chimeric FPR−$G_{\alpha i2}$ protein yielded results very different from those of the WT FPR. Whereas the phosphorylated WT FPR does not reconstitute with G proteins but does reconstitute with arrestins, the phosphorylated chimeric FPR−$G_{\alpha i2}$ protein displayed high-affinity agonist binding that was sensitive to the addition of GTPγS. This indicated that the linked G protein was capable of interacting with the phosphorylated chimeric receptor. The lack of a significant competitive interaction with exogenous arrestin suggests that the high local concentration of the linked G protein αi2 subunit can effectively compete with the added arrestin. To test whether the arrestin could in fact bind to the phosphorylated chimeric FPR−$G_{\alpha i2}$ protein in vitro, as had been observed in vivo, GTPγS was used to inhibit binding of the G protein αi2 subunit. Under these conditions, both the wild-type arrestin-2 and the high-affinity truncated form of arrestin-2 were capable of inducing a ligand affinity shift, confirming the phosphorylated status of the receptor. These results confirm that G proteins can interact with the phosphorylated form of the FPR but, given our previous results with exogenously added G proteins, which do not associate with the phosphorylated FPR at concentrations approximately 10-fold greater than their K_d for the unphosphorylated receptor, suggest that the affinity of the interaction is very low.

In this study, we have demonstrated for the first time that increasing the local concentration of the G protein αi2 subunit in the vicinity of a GPCR will facilitate the interaction of the G protein with the phosphorylated form of the receptor. Despite this, the chimeric FPR−$G_{\alpha i2}$ protein underwent normal desensitization in vivo, consistent with the requirement for an additional factor, arrestin, to block the G protein interaction completely. Arrestin binding to the chimeric FPR−$G_{\alpha i2}$ protein was observed in vivo but required the addition of GTPγS in vitro to compete effectively for binding with the linked G protein. This suggests that, in vivo, the high concentration of GTP relative to GDP allows binding of arrestin at times when the G protein is in an activated state and not able to interact functionally with the receptor. This is further supported by our observation of the complete lack of a functional interaction between GTP-bound G proteins and the FPR. These results raise the intriguing question as to the actual local concentrations of G proteins in the cell membrane, whether they are present at sufficiently high concentrations to interact with the FPR in its phosphorylated state, and whether this interaction with a phosphorylated receptor leads to G protein activation. Resolution of this question in vivo will require expression of the FPR in cells lacking arrestins, so that the confounding effects of arrestin binding can be eliminated.

Finally, the assembly of the fusion protein on particles leads to numerous novel possibilities. We have recently assembled the FPR−$G_{\alpha i2}$ fusion protein with epitope-tagged $\beta\gamma$ subunits attached to particles. This format is suitable for high-throughput flow cytometry including the screening of ternary complex assemblies, kinetics analyses of activation mechanisms, and drug discovery. The use of a receptor−G_α protein−GFP chimera (*43*) could further facilitate this process. The characterization of the functional properties of such fusion proteins at this current level of molecular and cellular detail is a prerequisite for meaningful mechanistic studies as well as drug discovery.

REFERENCES

1. Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., et al. (2001) *Science 291*, 1304−1351.
2. Ferguson, S. S., Zhang, J., Barak, L. S., and Caron, M. G. (1998) *Life Sci. 62*, 1561−1565.
3. Ferguson, S. S. (2001) *Pharmacol. Rev. 53*, 1−24.
4. Bhattacharya, M., Anborgh, P. H., Babwah, A. V., Dale, L. B., Dobransky, T., Benovic, J. L., Feldman, R. D., Verdi, J. M., Rylett, R. J., and Ferguson, S. S. (2002) *Nat. Cell Biol. 4*, 547−555.
5. Miller, W. E., and Lefkowitz, R. J. (2001) *Sci. STKE 2001*, E1.
6. Luttrell, L. M., Roudabush, F. L., Choy, E. W., Miller, W. E., Field, M. E., Pierce, K. L., and Lefkowitz, R. J. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 2449−2454.
7. Bennett, T. A., Maestas, D. C., and Prossnitz, E. R. (2000) *J. Biol. Chem. 275*, 24590−24594.
8. Bennett, T. A., Foutz, T. D., Gurevich, V. V., Sklar, L. A., and Prossnitz, E. R. (2001) *J. Biol. Chem. 276*, 49195−49203.
9. Key, T. A., Bennett, T. A., Foutz, T. D., Gurevich, V. V., Sklar, L. A., and Prossnitz, E. R. (2001) *J. Biol. Chem. 276*, 49204−49212.
10. Wilden, U., Hall, S. W., and Kuhn, H. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1174−1178.
11. Gibson, S. K., Parkes, J. H., and Liebman, P. A. (2000) *Biochemistry 39*, 5738−5749.
12. Gurevich, V. V., Dion, S. B., Onorato, J. J., Ptasienski, J., Kim, C. M., Sterne-Marr, R., Hosey, M. M., and Benovic, J. L. (1995) *J. Biol. Chem. 270*, 720−731.
13. Krupnick, J. G., Gurevich, V. V., and Benovic, J. L. (1997) *J. Biol. Chem. 272*, 18125−18131.
14. Allen, R. A., Jesaitis, A. J., Sklar, L. A., Cochrane, C. G., and Painter, R. G. (1986) *J. Biol. Chem. 261*, 1854−1857.
15. Snyderman, R., and Pike, M. C. (1984) *Annu. Rev. Immunol. 2*, 257−281.
16. Prossnitz, E. R., and Ye, R. D. (1997) *Pharmacol. Ther. 74*, 73−102.
17. Bokoch, G. M., Bickford, K., and Bohl, B. P. (1988) *J. Cell Biol. 106*, 1927−1936.
18. Prossnitz, E. R., Kim, C. M., Benovic, J. L., and Ye, R. D. (1995) *J. Biol. Chem. 270*, 1130−1137.
19. Hsu, M. H., Chiang, S. C., Ye, R. D., and Prossnitz, E. R. (1997) *J. Biol. Chem. 272*, 29426−29429.
20. Prossnitz, E. R. (1997) *J. Biol. Chem. 272*, 15213−15219.
21. Maestes, D. C., Potter, R. M., and Prossnitz, E. R. (1999) *J. Biol. Chem. 274*, 29791−29795.
22. Bertin, B., Freissmuth, M., Jockers, R., Strosberg, A. D., and Marullo, S. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 8827−8831.
23. Seifert, R., Lee, T. W., Lam, V. T., and Kobilka, B. K. (1998) *Eur. J. Biochem. 255*, 369−382.
24. Bertin, B., Strosberg, A. D., and Marullo, S. (1997) *Int. J. Cancer 71*, 1029−1034.
25. Bertin, B., Jockers, R., Strosberg, A. D., and Marullo, S. (1997) *Recept. Channels 5*, 41−51.
26. Seifert, R., Wenzel-Seifert, K., Lee, T. W., Gether, U., Sanders-Bush, E., and Kobilka, B. K. (1998) *J. Biol. Chem. 273*, 5109−5116.
27. Wise, A., Carr, I. C., Groarke, D. A., and Milligan, G. (1997) *FEBS Lett. 419*, 141−146.
28. Wise, A., Sheehan, M., Rees, S., Lee, M., and Milligan, G. (1999) *Biochemistry 38*, 2272−2278.
29. Carr, I. C., Burt, A. R., Jackson, V. N., Wright, J., Wise, A., Rees, S., and Milligan, G. (1998) *FEBS Lett. 428*, 17−22.
30. Milligan, G. (2000) *Trends Pharmacol. Sci. 21*, 24−28.
31. Wenzel-Seifert, K., Arthur, J. M., Liu, H. Y., and Seifert, R. (1999) *J. Biol. Chem. 274*, 33259−33266.
32. Seifert, R., Wenzel-Seifert, K., and Kobilka, B. K. (1999) *Trends Pharmacol. Sci. 20*, 383−389.

33. Gurevich, V. V., and Benovic, J. L. (2000) *Methods Enzymol. 315*, 422−437.
34. Sklar, L. A., Oades, Z. G., Jesaitis, A. J., Painter, R. G., and Cochrane, C. G. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7540−7544.
35. Cobbold, P. H., and Rink, T. J. (1987) *Biochem. J. 248*, 313−328.
36. Prossnitz, E. R., Schreiber, R. E., Bokoch, G. M., and Ye, R. D. (1995) *J. Biol. Chem. 270*, 10686−10694.
37. Bennett, T. A., Key, T. A., Gurevich, V. V., Neubig, R., Prossnitz, E. R., and Sklar, L. A. (2001) *J. Biol. Chem. 276*, 22453−22460.
38. Jesaitis, A. J., Tolley, J. O., Bokoch, G. M., and Allen, R. A. (1989) *J. Cell Biol. 109*, 2783−2790.
39. Bommakanti, R. K., Dratz, E. A., Siemsen, D. W., and Jesaitis, A. J. (1994) *Biochim. Biophys. Acta 1209*, 69−76.
40. Waldhoer, M., Wise, A., Milligan, G., Freissmuth, M., and Nanoff, C. (1999) *J. Biol. Chem. 274*, 30571−30579.
41. Jesaitis, A. J., Bokoch, G. M., Tolley, J. O., and Allen, R. A. (1988) *J. Cell Biol. 107*, 921−928.
42. Sterne-Marr, R., Gurevich, V. V., Goldsmith, P., Bodine, R. C., Sanders, C., Donoso, L. A., and Benovic, J. L. (1993) *J. Biol. Chem. 268*, 15640−15648.
43. Bevan, N., Palmer, T., Drmota, T., Wise, A., Coote, J., Milligan, G., and Rees, S. (1999) *FEBS Lett. 462*, 61−65.

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