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Experimental Evidence for Lack of Homodimerization of the G Protein-Coupled Human *N*-Formyl Peptide Receptor[1]

Jeannie M. Gripentrog, Katrin P. Kantele, Algirdas J. Jesaitis, and Heini M. Miettinen[2]

A large number of G protein-coupled receptors have been shown to form homodimers based on a number of different techniques such as receptor coimmunoprecipitation, cross-linking, and fluorescence resonance energy transfer. In addition, functional assays of cells coexpressing a mutant receptor with a wild-type receptor have shown receptor phenotypes that can best be explained through dimerization. We asked whether the human neutrophil *N*-formyl peptide receptor (FPR) forms dimers in Chinese hamster ovary cells by coexpressing wild-type FPR with one of two mutants: D71A, which is uncoupled from G protein, and N297A, which has a defect in receptor phosphorylation and endocytosis. Experiments measuring chemotaxis, ligand-induced release of intracellular calcium, and p42/44 mitogen-activated protein kinase activation did not show an inhibitory effect of the coexpressed FPR D71A mutant. Coexpressed wild-type receptor was efficiently internalized, but failed to correct the endocytosis defects of the D71A and the N297A mutants. To explore the possibility that the mutations themselves prevented dimerization, we examined the coimmunoprecipitation of differentially epitope-tagged FPR. Immunoprecipitation of hemagglutinin-tagged FPR failed to coimmunoprecipitate coexpressed c-*myc*-tagged FPR and vice versa. Together, these data suggest that, unlike many other G protein-coupled receptors, FPR does not form homodimers. ***The Journal of Immunology*****, 2003, 171: 3187–3193.**

The human *N*-formyl peptide receptor (FPR)[3] belongs to the large family of seven transmembrane domain G protein-coupled receptors (GPCRs). FPR is expressed primarily by neutrophils and monocytes and mediates a number of important host defense functions such as chemotactic migration and killing of microorganisms through oxidative burst and phagocytosis (for review, see Ref. 1). FPR binds bacterial *N*-formylated peptides, such as fMLF, which is the prototypic formylated peptide purified from *Escherichia coli* cultures (2). More recently, certain nonformylated peptides have been observed to bind to and activate neutrophils through FPR (3–7).

We have previously examined various functions of wild-type FPR and a number of FPR mutants in stably transfected Chinese hamster ovary (CHO) cells. Although these cells do not exhibit an oxidative burst, other cellular functions characteristic of neutrophils can be observed. These include fMLF-induced calcium release, FPR phosphorylation and endocytosis, activation of p42/44 mitogen-activated protein kinase (MAPK), and chemotactic migration (8, 9).

Because a wide variety of responses appear to be differentially regulated through the same receptor, attention has recently been focused on early receptor events that could have consequences for specific signal transduction pathways. A number of studies have suggested that many GPCRs form homo- and heterodimers and that the dimerization contributes to modulation of receptor function (10). Dimer-forming receptors include (but are not limited to) β_2-adrenergic receptor (11), the δ opioid receptor (12), somatostatin receptors (13), CXCR4 (14), CCR2 (15), and CCR5 (16, 17). The last three of these are chemokine receptors that mediate chemotaxis toward their ligands in a manner similar to FPR. Because of the close structural and functional similarity of dimer-forming chemotactic receptors to FPR, we sought to determine whether FPR also forms dimers.

The first studies on GPCR dimerization were conducted by coimmunoprecipitation of differentially epitope-tagged receptors. More recently, various functional studies have been employed to examine dimerization. For example, expression of a loss-of-function mutant of CCR2 was shown to block the signaling through wild-type CCR2 by dimerization (15). Similarly, a D63N mutant of the human platelet-activating factor receptor that did not detectably bind G protein resulted in a constitutively active receptor phenotype when coexpressed with wild-type receptor (18).

In the current study, we coexpress with wild-type FPR a mutant (D71A) that is uncoupled from G protein. The D71A mutation is analogous to the D63N mutation in the human platelet-activating factor receptor. To examine the possible dimerization of the D71A mutant with wild-type FPR, we examined ligand-induced intracellular calcium release, chemotaxis, and activation of p42/44 MAPK. Another FPR mutant, N297A, which has a defect in receptor endocytosis, was also expressed with wild-type receptor to examine its effect on the endocytosis of the wild-type receptor. In addition, we conducted coimmunoprecipitation analysis of FPR with two different amino-terminal epitope tags. The combined results from these studies suggest that FPR does not form homodimers.

Materials and Methods

Plasmids and cell culture

Construction and characterization of CHO cells expressing FPR wild type (isoform 26), FPR D71A, and FPR N297A have been described previously (9, 19, 20). The enhanced green fluorescent protein (EGFP) cDNA was amplified by PCR from pEGFP-N1 (Clontech Laboratories, Palo Alto, CA)

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[3] Abbreviations used in this paper: FPR, *N*-formyl peptide receptor; GPCR, G protein-coupled receptor; CHO, Chinese hamster ovary; MAPK, mitogen-activated protein kinase; EGFP, enhanced green fluorescent protein; HA, hemagglutinin; GABA, γ-aminobutyric acid.

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using Ready-to-Go PCR beads (Amersham Biosciences, Arlington Heights, IL). The stop-codon in wild-type and mutant FPRs was mutated into a *Bam*HI site that was used for ligation of the EGFP insert. The amino acid sequence encoded by the splice site was ..VELQAK/*DPPVAT*/MVSKG.., with the italicized amino acids derived from the pEGFP-N1 multiple cloning site. FPR-EGFP, D71A-EGFP, and N297A-EGFP constructs were subcloned into the constitutive expression vectors pBGSA (21) and/or pBZSA (H. M. Miettinen, unpublished data) to allow selection of CHO transfectants with G418 and Zeocin (or Bleocin), respectively. The c-*myc*-FPR and hemagglutinin (HA)-FPR constructs were made by PCR. The forward primers contained the nucleotide sequences coding for the 10-aa c-*myc* epitope (EQKLISEEDL) or the 9-aa HA epitope (YPYDVP DYA) plus 7 amino-terminal amino acids of FPR (ETNSSLP). The reverse primer contained the nucleotide sequence encoding nine amino acids of FPR spanning a unique *Nco*I site. The PCR products were first ligated into pGEM-T Easy (Promega, Madison, WI), digested with *Eco*RI and *Nco*I, and ligated into the corresponding site of digested pBGSA-FPR. The constructs were confirmed by sequencing. CHO cells were transfected and cultured as previously described (19).

Abs and reagents

The polyclonal Ab against the C-terminal 12 aa of FPR has been described previously (19). The MYC 1-9E10.2 hybridoma clone producing the anti-c-*myc* Ab was purchased from American Type Culture Collection (Manassas, VA) (22). The Ab was purified from the hybridoma culture supernatant by ammonium sulfate precipitation. The agarose-conjugated anti-c-*myc* 9E10 Ab was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), the mouse anti-HA 16B12 Ab was purchased from Covance Research Products (Berkeley, CA), and the rabbit anti-HA and rabbit anti-c-*myc* Abs were obtained from Sigma-Aldrich (St. Louis, MO). Other reagents were from Sigma-Aldrich, unless indicated otherwise.

Calcium assay

Release of intracellular calcium was analyzed as described previously (9). Briefly, transfected CHO cells were incubated with 2.5 μM fura 2-acetoxymethyl ester (Molecular Probes, Eugene, OR) for 45 min at 37°C. Cells were centrifuged and resuspended in PBS containing 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$ and 5% (v/v) FBS, and left at room temperature until the assay (5–60 min). Each measurement contained $\sim 5 \times 10^6$ CHO cells in a volume of 1 ml. Continuous fluorescence measurements of calcium-bound and free fura 2 were made at 37°C with a double excitation monochromator fluorescence spectrofluorometer (Photon Technology International, Monmouth Junction, NJ) with excitation at 340 and 380 nm and emission at 510 nm. Cells were exposed to 100 nM fMLF, and 10 μM ATP was added later as a heterologous ligand to provide a standard stimulus for calcium mobilization.

Chemotaxis assay

Chemotaxis assays were conducted essentially as described previously (8). Briefly, CHO transfectants at 3×10^5 cells in 300 μl were added to 6.5-mm diameter Transwell inserts of 8-μm pore size (Corning Costar, Cambridge, MA). Five hundred microliters of serum-free medium with or without fMLF at various concentrations were placed in the wells of a 24-well culture plate. The Transwell inserts containing the cells were moved into the wells, and chemotaxis was allowed to proceed for 4 h at 37°C. Cells on the upper face of each insert were removed with cotton swabs; cells that adhered to the underside of each filter were fixed with 2.5% paraformaldehyde in PBS, stained with hematoxylin Gill's stain, and quantified using an image analyzer (M4 True Color Image Analysis System; Imaging Research, St. Catharines, Ontario, Canada).

MAPK assay

CHO transfectants were incubated for 5 min with or without 10 nM fMLF and lysed with boiling Laemmli sample buffer (12.5 mM Tris-HCl, 2% SDS, 7.5% glycerol, 20 mM DTT, and 0.01% bromphenol blue, pH 6.8). The incubation time was based on previous experiments that showed maximal p42/44 MAPK activation at 5 min for FPR-transfected cells (20). Extract corresponding to $\sim 1 \times 10^5$ cells was run on a 10% SDS-polyacrylamide gel. Proteins were electrophoretically transferred to nitrocellulose and p42 MAPK was detected using either a mouse mAb against human p42/44 MAPK phosphorylated on Thr202 and Tyr204 or a rabbit polyclonal Ab against total human p42/44 MAPK (Cell Signaling Technology, Beverly, MA). The proteins were detected by ECL (Pierce, Rockford, IL) using HRP-conjugated secondary Ab (Jackson ImmunoResearch Laboratories, West Grove, PA). The intensity of the bands on the films was quantified using ImageQuant software (Amersham Biosciences, Arlington Heights, IL) after scanning with a Hewlett-Packard Scanjet IIcx scanner (Palo Alto, CA).

Confocal microscopy

The CHO double transfectants were incubated on ice with anti-c-*myc* or anti-HA Abs to label surface-expressed epitope-tagged FPR, as indicated in the figure legends. The cells were moved to 37°C and incubated for 2 h in the presence or absence of 1 μM fMLF. Cells were fixed in 2.5% paraformaldehyde/PBS, blocked, and permeabilized in 0.2% gelatin and 0.01% saponin/PBS. The secondary Abs were Alexa488- and Alexa568-conjugated goat anti-rabbit and goat anti-mouse Abs (Molecular Probes) at 1/400

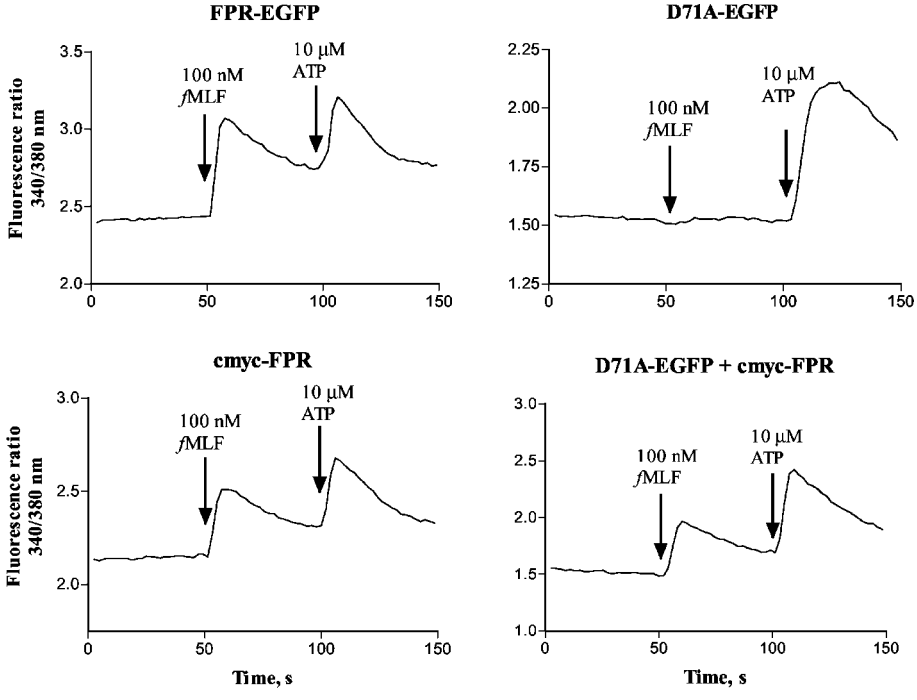

FIGURE 1. Intracellular calcium release in CHO transfectants expressing FPR-EGFP, D71A-EGFP, c-*myc*-FPR, or a combination of D71A-EGFP and c-*myc*-FPR. Cells loaded with fura 2-acetoxymethyl ester were induced to release intracellular calcium by the addition of 100 nM fMLF. After 50 s, 10 μM ATP was added as a control to confirm that the cells were still responsive.

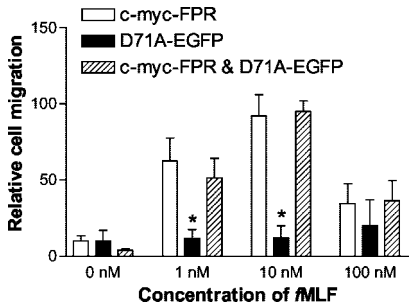

FIGURE 2. Chemotaxis of CHO transfectants toward various concentrations of fMLF. Cells were allowed to migrate toward 0, 1, 10, or 100 nM fMLF for 4 h. Cells that had migrated through 8-μm pores and attached to the other side of the semipermeable membrane were fixed, stained, and quantified using an image analyzer. The chemotaxis is shown as the relative surface area of the cells on the filter. The migration of cells expressing c-*myc*-FPR alone or cells expressing both c-*myc*-FPR and D71A-EGFP are almost identical, suggesting that the coexpressed D71A mutant does not attenuate the cells' ability to migrate toward a gradient of fMLF. The results represent means ± SEM from three separate experiments. *, $p < 0.05$.

dilutions. Confocal scans were conducted sequentially using either the argon or the krypton laser and the images were merged using Imaris 3 software. The picture layouts were created using Adobe Photoshop (Adobe Systems, San Jose, CA) and Microsoft PowerPoint software (Microsoft, Redmond, WA).

Immunoprecipitation and Western blot analysis

Confluent monolayers of double-transfected CHO cells on 60-mm dishes were incubated for 10 min with 1 μM fMLF, rinsed with cold PBS, and extracted for 1 h on ice with 700 μl of 0.5% *n*-dodecyl-D-maltoside, 25 mM Tris-HCl (pH 7.4), 140 mM NaCl, 2 mM EDTA, 1 mM PMSF, and Sigma-Aldrich protease inhibitor mixture (17). Cell extracts were centrifuged (20,000 × *g*, 15 min, 4°C) and supernatants were precleared with nonrelevant mouse IgG and protein G-Sepharose. Immunoprecipitations were conducted overnight at 4°C with 10 μl of agarose-anti-c-*myc* 9E10 Ab or 5 μl of mouse anti-HA 16B12 Ab followed by protein G-Sepharose. Proteins were separated on a 7.5% SDS-polyacrylamide gel, transferred to a nitrocellulose filter (Amersham Biosciences), and Western blot analysis was conducted using rabbit anti-FPR Ab, rabbit anti-HA Ab, or rabbit anti-c-*myc* Ab, followed by peroxidase-conjugated secondary Ab and ECL.

Results

Previous reports suggest that dimerization of a functional wild-type GPCR with a nonfunctional mutant may result in changes in ligand binding, receptor endocytosis, and signaling properties. If the mutant receptor is dominant negative, the phenotype in cells expressing both wild-type and mutant receptor will be similar to cells expressing only the mutant receptor. The reverse is also possible, where the dimerization of the wild-type receptor with the mutant receptor results in a fully functional receptor phenotype. We decided to take advantage of this phenomenon to examine whether FPR forms homodimers.

D71A mutant does not inhibit calcium release induced by wild-type FPR

An FPR mutation with an aspartic acid to alanine substitution in position 71 (D71A; second transmembrane domain) results in uncoupling from G protein and a defect in signaling, receptor endocytosis and receptor-mediated chemotaxis (9, 20, 23). To examine the effect of D71A on wild-type FPR, cells stably expressing D71A with EGFP fused to its C terminus (D71A-EGFP) were transfected with a vector encoding c-*myc*-FPR and a Zeocin resistance gene for selection. A clone with high expression levels of both D71A-EGFP and c-*myc*-FPR (based on fluorescence intensities; see Fig. 4) was propagated for future experiments.

Homodimerization of wild-type FPR and D71A would be expected to result in a marked decrease in ligand-induced release of intracellular calcium if D71A is a dominant negative mutant. However, if D71A is a recessive mutant, we would not expect to see a change in calcium release, compared with cells expressing wild-type FPR alone. In a third scenario, if dimerization does not take place, release of intracellular calcium through the wild-type receptor would be observed.

First, we confirmed our earlier finding that the D71A mutation in FPR prevents fMLF-induced calcium release (Fig. 1). To confirm that the C-terminal EGFP itself did not affect calcium release, we examined the calcium release using cells expressing FPR-EGFP (Fig. 1). Similarly, the N-terminal c-*myc* tag did not affect calcium release through wild-type FPR. When cells expressing both D71A-EGFP and c-*myc*-FPR were exposed to fMLF, intracellular calcium release was comparable to that seen in cells expressing c-*myc*-FPR alone, suggesting that the D71A mutation

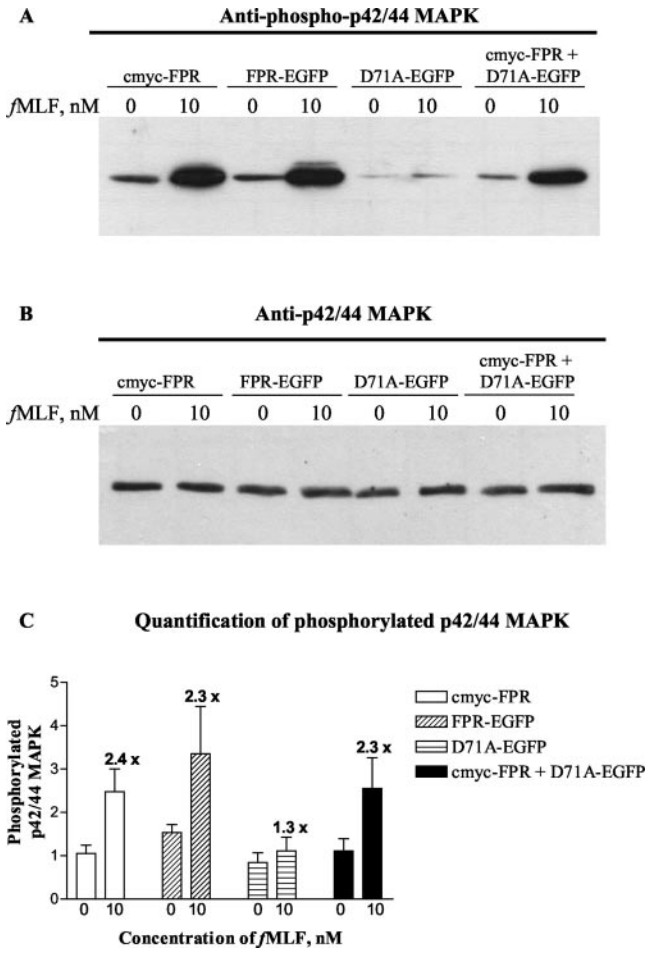

FIGURE 3. Activation of p42/44 MAPK in FPR transfectants. Cells expressing c-*myc*-FPR, FPR-EGFP, D71A-EGFP, and both c-*myc*-FPR and D71A-EGFP were exposed to 10 nM fMLF for 5 min. Cells were harvested and the amount of phosphorylated and total p42/44 MAPK was quantified by Western blotting. *A*, Western blot probed with a mAb against p42/44 MAPK phosphorylated on Thr202 and Thr204. *B*, Western blot of the same samples as in *A* probed with a mAb against total p42/44 MAPK. *C*, Quantification of phosphorylated p42/44 MAPK by densitometry from three different experiments. The limited dynamic range of the film does not allow accurate quantitative comparison of the signals, but an increase in phosphorylation of at least 2-fold is evident in cells expressing FPR alone or in combination with D71A, whereas cells expressing D71A alone show a negligible increase.

does not confer a dominant negative phenotype. Thus, the results point toward two different possibilities: 1) Wild-type FPR can correct the mutant phenotype or 2) no dimers form between the D71A mutant and wild-type FPR, allowing normal signaling through the wild-type receptor. A third possibility, which will be discussed in more detail below, is that the D71A mutation prevents the mutant receptor from forming dimers with the wild-type receptor.

D71A mutant does not affect chemotactic migration of CHO cells coexpressing FPR

Another downstream effect of FPR activation is migration up a concentration gradient of ligand. We have previously shown that cells expressing D71A do not migrate toward fMLF (9). To examine whether coexpression of D71A with wild-type FPR alters the chemotaxis efficiency, CHO double transfectants were allowed to migrate toward various concentrations of fMLF. The results show that the double transfectants migrated toward fMLF with a similar concentration dependence as CHO cells expressing c-*myc*-FPR alone (Fig. 2). These results lend further support to our calcium release data suggesting that in the event of receptor dimerization, D71A is not a dominant negative mutant.

D71A mutant does not attenuate the activation of p42/44 MAPK through wild-type FPR

As a downstream signaling event, p42/44 MAPK is activated upon ligand binding to FPR. We have previously shown that 1–100 nM fMLF does not result in activation of p42/44 MAPK through the D71A mutant, whereas a 2- to 4-fold increase in phosphorylated p42/44 MAPK can be detected in cells expressing wild-type FPR (9). To examine whether the D71A mutant affects the level of p42/44 MAPK phosphorylation when coexpressed with wild-type FPR, we compared the amount of phosphorylated p42/44 MAPK in these cells to cells expressing wild-type FPR alone. For these experiments, we again used the double-transfected CHO cells expressing c-*myc*-FPR and D71A-EGFP. To confirm that the C-terminal EGFP does not prevent the activation of p42/44 MAPK, we first showed that ligand binding to FPR-EGFP resulted in similar phosphorylation of p42/44 MAPK as seen with wild-type FPR where there was a greater than 2-fold increase (Fig. 3*A*). When cells coexpressing c-*myc*-FPR and D71A-EGFP were exposed to fMLF, a similar increase in phosphorylation of p42/44 MAPK activation was detected as seen in cells expressing c-*myc*-FPR alone. The D71A-EGFP mutant alone showed no statistically significant increase in phosphorylation. Thus, these results again confirm that the D71A mutant does not attenuate the signaling through wild-type FPR.

The endocytosis-defective D71A and N297A mutants remain on the cell surface and are not coendocytosed with wild-type FPR

All experiments described above fail to differentiate between the two possibilities that wild-type FPR forms dimers with the D71A mutant, restoring wild-type phenotype, or that FPR does not form dimers/oligomers. To distinguish between these two possibilities, we examined whether the endocytosis-deficient D71A-EGFP is coendocytosed with wild-type FPR upon ligand binding. Cells were incubated on ice with anti-c-*myc* Ab to label surface-expressed c-*myc*-FPR. Cells were then warmed up to 37°C and incubated for 2 h with or without 1 μM fMLF. To control for the possibility that the C-terminal EGFP may prevent receptor endocytosis, we also coexpressed FPR-EGFP with c-*myc*-FPR. As seen in Fig. 4, both FPR-EGFP and c-*myc*-FPR were endocytosed in the

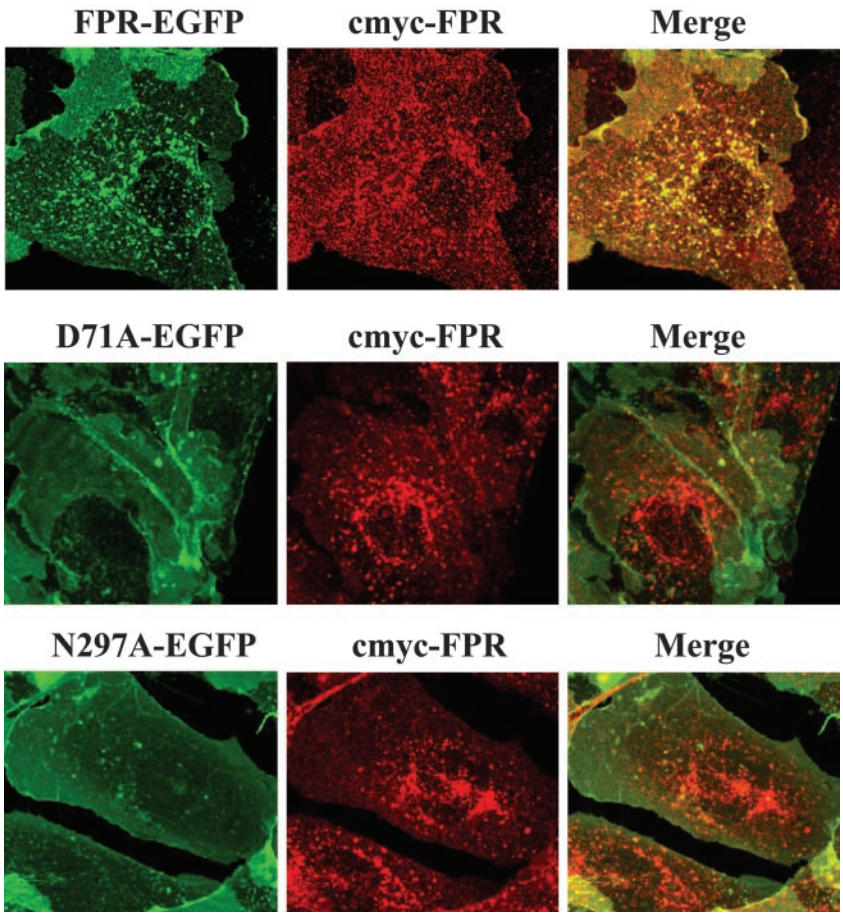

FIGURE 4. Analysis of receptor endocytosis in CHO cells coexpressing FPR-EGFP, D71A-EGFP, or N297A-EGFP with c-*myc*-FPR. Cells were incubated for 1 h on ice with mouse anti-c-*myc* Ab to label the c-*myc* epitope tag. The cells were then warmed to 37°C and endocytosis was allowed to proceed for 2 h in the presence or absence of 1 μM fMLF. Cells were fixed, permeabilized, and incubated with Alexa568 goat anti-mouse Ab to detect c-*myc*-FPR.

presence of fMLF and appeared to colocalize in vesicular structures. However, in cells expressing D71A-EGFP and c-*myc*-FPR, addition of ligand did not alter the distribution of D71A-EGFP, whereas c-*myc*-FPR was largely endocytosed (Fig. 4). Thus, no evidence for dimerization between D71A-EGFP and c-*myc*-FPR could be detected based on this endocytosis assay.

Since these data do not rule out the possibility that the mutation itself prevents dimerization, we also examined the endocytosis behavior of another FPR mutant, N297A. This mutant is capable of binding ligand and activating G protein, but has a defect in receptor phosphorylation and receptor endocytosis (9). As shown with D71A-EGFP, coexpression of N297A-EGFP with c-*myc*-FPR did not result in coendocytosis of the mutant receptor with the wild-type receptor (Fig. 4). Thus, these two FPR mutants do not appear to form dimers with c-*myc*-FPR.

Differentially epitope-tagged FPRs do not coimmunoprecipitate each other

Since the experiments described above examined the dimerization between mutant and wild-type FPR, we also decided to examine the dimerization of fully functional, epitope-tagged wild-type FPR. The most commonly used epitope tags in dimerization studies are FLAG and c-*myc*. We were unsuccessful in stably expressing FLAG-tagged FPR due to intracellular retention of a large proportion of the FLAG-FPR, possibly due to misfolding. We therefore produced an HA-tagged FPR that was successfully coexpressed with c-*myc*-FPR. Cells expressing HA-FPR and c-myc-FPR bound *f*-Nle-Leu-Phe-Nle-Tyr-Lys-fluorescein with an affinity similar to the cells expressing the wild-type receptor (K_D = 5 and 6 nM, respectively), suggesting that the epitope tags do not interfere with ligand binding (19). Fig. 5*A* shows the cell surface expression of the two receptors by immunofluorescence microscopy using monoclonal and polyclonal Abs against the epitope tags. Receptor expression and Abs were also characterized by Western blot analysis using CHO membranes from untransfected CHO cells (as negative control) and CHO cells coexpressing c-*myc*-FPR and HA-FPR. The mAbs gave less background staining than the polyclonal Abs and were therefore used for the immunoprecipitation experiments (described below), whereas the polyclonal Abs were used to identify the immunoprecipitated proteins by Western blotting (Fig. 5*B*).

Since many GPCRs require ligand binding to form homodimers (or heterodimers), we incubated the CHO c-*myc*-FPR/HA-FPR double transfectants with fMLF before cell extraction. The cell extractions and immunoprecipitations were conducted essentially as described by Chelli and Alizon (17), who examined CCR5 dimerization by coimmunoprecipitation. The decision to follow this method was based on the structural and functional similarity between CCR5 and FPR. CHO cells coexpressing HA-FPR and c-*myc*-FPR were extracted with detergent and the extracts were immunoprecipitated with mouse mAbs against each epitope tag. Successful immunoprecipitation was documented by immunoblot analysis using a polyclonal Ab against FPR (Fig. 6*A*). However, when the blot was probed with a polyclonal Ab against c-*myc*, only protein immunoprecipitated with mouse anti-c-*myc* Ab could be

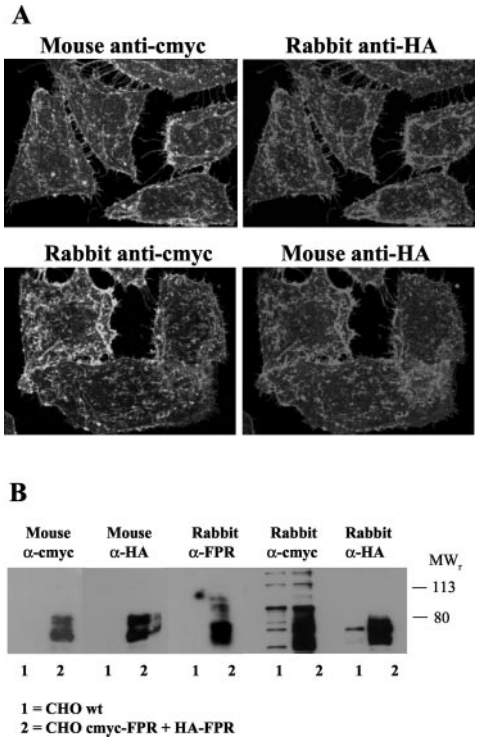

FIGURE 5. *A,* Characterization of cell surface expression of c-*myc*-FPR and HA-FPR in double-transfected CHO cells by immunofluorescence microscopy. Cells on coverslips were incubated for 1 h on ice with mouse anti-c-*myc* and rabbit anti-HA or with rabbit anti-c-*myc* and mouse anti-HA, rinsed, fixed, and incubated with Alexa488- and Alexa568-conjugated goat anti-rabbit and goat anti-mouse Abs. Corresponding pairs of fluorescence micrographs are shown. *B,* Characterization of Abs against the epitope tags and anti-FPR Ab by Western blot analysis. Membranes from nontransfected CHO cells and CHO cells coexpressing c-*myc*-FPR and HA-FPR were extracted with Laemmli sample buffer, the proteins were separated on an SDS-polyacrylamide gel, transferred onto nitrocellulose, and probed with the various Abs as indicated, followed by peroxidase-conjugated secondary Abs and ECL. wt, Wild type.

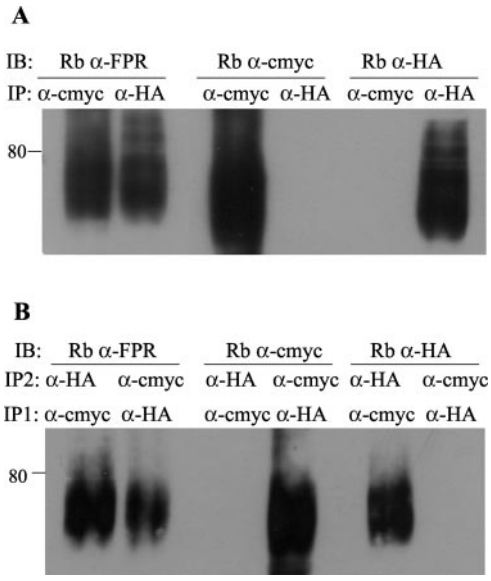

FIGURE 6. *A,* Coimmunoprecipitation and Western blot analysis of CHO cells coexpressing c-*myc*-FPR and HA-FPR. Cells were incubated for 10 min with 1 μM fMLF and extracted with detergent; lysates were subjected to immunoprecipitation (IP) with mouse anti-c-*myc* or mouse anti-HA Ab. The immunoprecipitated c-*myc*-FPR and HA-FPR were detected on nitrocellulose using rabbit anti-FPR, rabbit anti-c-*myc*, and rabbit anti-HA Abs (IB). *B,* Secondary immunoprecipitation of supernatants. The supernatant remaining after immunoprecipitation with anti-c-*myc* Ab was immunoprecipitated with anti-HA Ab (IP2), and the supernatant remaining after immunoprecipitation with anti-HA Ab was immunoprecipitated with anti-c-*myc* Ab (IP2). Immunoblot analysis (IB) was conducted with the same Abs as above. Rb, rabbit.

detected, suggesting that immunoprecipitation of HA-FPR did not result in coimmunoprecipitation of c-*myc*-FPR. Similarly, when the proteins immunoprecipitated with anti-c-*myc* and anti-HA were detected by immunoblot analysis using a polyclonal Ab against the HA epitope, only the receptor immunoprecipitated with mouse anti-HA Ab was detectable (Fig. 6*A*). To confirm that the other epitope-tagged FPR remained in the supernatant after the immunoprecipitation, we performed a second round of immunoprecipitations. HA-FPR could be immunoprecipitated from the remaining supernatant after immunoprecipitation with anti-c-*myc* Ab and vice versa as shown in Fig. 6*B*. Finally, no change in immunoprecipitation results was obtained when the cross-linking reagent disuccinimidyl suberate was used before the cell extraction (data not shown). Thus, these results suggest that c-*myc*-FPR and HA-FPR do not form dimers.

Discussion

The formation of receptor homo- and heterodimers has been proposed for a number of GPCRs based on cross-linking, coimmunoprecipitation and fluorescence resonance energy transfer studies or on functional complementation of mutant and chimeric receptors (for review, see Refs. 10 and 24). Dimerization may have important functional implications for receptor function. The most intriguing examples can be found within the family of neurotransmitter receptors: heterodimerization of γ-aminobutyric acid ($GABA_B$) receptor 1 ($GABA_BR1$) with $GABA_BR2$ is required not only for high-affinity binding of GABA and signal transduction, but also for efficient receptor transport to the cell surface after synthesis (for review, see Ref. 25). Receptors for dopamine (D2R) and somatostatin (SSTR5) form heterodimers in the presence of neurotransmitter, resulting in enhanced ligand binding and functional activity (26).

The functional effect of homodimerization has been studied using peptides that block dimerization and by expression of nonfunctional mutants with either wild-type receptor or with a receptor containing a different mutation. A peptide coding the sixth transmembrane domain of β_2-adrenergic receptor blocks dimerization and decreases agonist-mediated signal transduction, suggesting that the dimerization activates G protein (11). The requirement for dimer formation in signal transduction has also been illustrated using a signaling-defective mutant of α-mating factor receptor in yeast: expression with wild-type receptor attenuated the signaling efficiency. On the other hand, the endocytosis defect of the mutant receptor was corrected by coexpressing wild-type receptor, suggesting that the mutant receptor did not exhibit a dominant phenotype (27).

To examine the possibility that FPR forms homodimers, we have taken advantage of a mutant FPR with an aspartic acid to alanine substitution in position 71, causing a defect in G protein-coupling, signaling, receptor endocytosis, and chemotaxis. By coexpressing this mutant with wild-type FPR, we show that the mutant does not attenuate the intracellular calcium release in response to fMLF (Fig. 1). Furthermore, cells expressing both D71A and wild-type FPR exhibit similar efficiency in chemotaxis and activation of p42/44 MAPK as cells expressing wild-type FPR alone (Figs. 2 and 3). Since dimerization also results in coendocytosis of the dimerization partners (27), we next examined whether the endocytosis-defective D71A mutant is endocytosed with wild-type FPR upon ligand binding. Our results show that when the double transfectants are exposed to fMLF, wild-type FPR is endocytosed, whereas D71A remains on the cell surface for up to 2 h (Fig. 4). Similarly, another endocytosis-defective mutant of FPR, N297A, remained on the cell surface whereas c-*myc*-FPR was endocytosed (Fig. 4).

Since the mutations themselves might be able to prevent receptor dimerization, we also examined the dimerization of epitope-tagged wild-type FPR. This approach has been used previously to show homo- and heterodimerization of a number of GPCRs, such as adrenergic receptors (28, 29), opioid receptors (30, 31), and chemokine receptors (17). Since dimerization studies have not previously been conducted with chemoattractant receptors, we chose to follow the procedure of Chelli and Alizon (17) who showed dimerization of wild-type and mutant CCR5 chemokine receptors. Our results show no dimerization under these conditions, even after extended chemiluminescence exposure times (Fig. 6). Since the expression levels of HA-FPR and c-*myc*-FPR were comparable based on immunofluorescence staining and Western blot analysis, we would have expected ~25% of the receptors to form c-*myc*-FPR/HA-FPR dimers. This amount of dimer should have been readily detectable on the Western blots. However, no coimmunoprecipitating product could be visualized. Together these results show that under these experimental conditions measuring ligand-induced intracellular calcium release, chemotaxis, p42/44 MAPK activation, receptor endocytosis, and receptor coimmunoprecipitation, no convincing evidence for homodimerization of FPR could be obtained.

Previous studies of chemoattractant and chemokine receptors in neutrophils have shown that FPR is dominant over receptors that bind C5a, IL-8, and Gro-α (32). It has been postulated that this dominance of FPR may ensure that the signals from microbial pathogens can override host-derived recruitment signaling through chemokine receptors (33). Since it has been shown that chemokine receptors modulate their ligand binding and signaling through heterodimerization (34), it remains to be determined whether the regulation of the classical chemoattractant receptors is likewise regulated through receptor heterodimerization.

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