A novel fluorescent photoaffinity cross-linking probe, formyl-Met-p-benzoyl-L-phenylalanine-Phe-Tyr-Lys-e-N-fluorescein (fMBpaFYK-fl), was synthesized and used to identify binding site residues in recombinant human phagocyte chemotractant formyl peptide receptor (FPR). After photoactivation, fluorescein-labeled membranes from Chinese hamster ovary cells were solubilized in octylglucoside and separated by tandem anion exchange and gel filtration chromatography. A single peak of fluorescence was observed in extracts of FPR-expressing cells that was absent in extracts from wild type controls. Photolabeled Chinese hamster ovary membranes were cleaved with CNBr, and the fluorescent fragments were isolated on an antifluorescein immunoadfinity matrix. Matrix-assisted laser desorption ionization mass spectrometry identified a major species with mass = 1754, consistent with the CNBr fragment of fMBpaFYK-fl cross-linked to Val-Arg-Lys-Ala-Hse (an expected CNBr fragment of FPR, residues 83–87). This peptide was further cleaved with trypsin, repurified by antifluorescein immunoadfinity, and subjected to matrix-assisted laser desorption ionization mass spectrometry. A tryptic fragment with mass = 1582 was observed, which is the mass of fMBpaFYK-fl cross-linked to Val-Arg-Lys (FPR residues 83–85). Derivatization of these residues with mass = 1754, consistent with the CNBr fragment of fMBpaFYK-fl cross-linked to Val-Arg-Lys-Ala-Hse, results in a putative second transmembrane-spanning region of FPR near the extracellular surface. A 3D model of FPR is presented, which accounts for intramembrane, site-directed mutagenesis results (Miettinen, H. M., Mills, J., Gripentrog, J., Dratz, E. A., Granger, B. L., and Jesaitis, A. J. (1997) J. Immunol. 159, 4045–4054) and the photochemical cross-linking data.

The phagocyte chemotactic receptors, including the formyl peptide receptor (FPR), 1 the lipoxin A4 receptor, the C5a receptor, the platelet-activating factor receptor, and the interleukin-8 receptor are involved in inflammation and are all members of the G protein-coupled receptor (GPCR) superfamily. Among the most studied in this inflammatory receptor family is neutrophil FPR (1). FPR binds N-formyl peptides, such as formyl-Met-Leu-Phe (fMLF), with nanomolar affinity (2). Such N-formyl peptides are indicators of the presence of bacteria (3) or damage to host cell mitochondria (4, 5). Binding of N-formyl peptides to FPR thus provides phagocytes with signals for infection or injury and results in activation of chemotaxis and other host defensive processes including lysosomal enzyme secretion, stimulation of production of inflammatory mediators, and generation of superoxide.

The effects of amino acid substitutions and modifications of fMLF peptides on binding to FPR and activation have been studied extensively (6–8). The formyl group, the methionine at position 1, and phenylalanine at position 3 have been shown to be necessary for high affinity binding. Decarboxylation of the C-terminal phenylalanine markedly reduces activity, but esters or amides of this residue or peptides with C-terminal amino acid additions exhibit similar activity to the tripeptide with the free acid. None of the fMLF functional groups have been shown to be absolutely essential for activity but rather they appear to individually contribute to the overall free energy of binding.

Chemical and photoaffinity cross-linking of fMLF analogs to FPR has been achieved by a number of groups (9–12). However, none of these studies have identified the site of labeling. The residue in the second position of N-formylated peptides appears to be the most tolerant of modification (6, 13) and both of the flanking residues are critical for high affinity binding (14); so the second residue was chosen to accommodate the photoreactive amino acid benzoylphenylalanine (Bpa). Bpa is chemically stable in the absence of photoexcitation, can be directly introduced into peptide ligands by solid phase peptide synthesis, and has been photocross-linked into several peptide receptors (15). Here we report that a fluorescent photoaffinity analog of fMLF, formyl-Met-p-benzoyl-l-phenylalanine-Phe-Tyr-Lys-e-N-fluorescein (fMBpaFYK-fl), efficiently photocross-links to FPR residues 83–85. Derivatization of these residues in FPR by Bpa supports recent site-directed mutagenesis stud-
ies predicting that the formyl peptide binding site of FPR lies within the transmembrane spanning region, near the transmembrane-extracellular interface of the receptor (17).

MATERIALS AND METHODS

Peptide Synthesis—Met-p-Benzoyl-l-phenylalanine-Phe-Tyr-Lys was synthesized from Fmoc (N(9-fluorenyl)methoxycarbonyl) amino acids using a Milligen 9050 peptide synthesizer and purified by reverse phase HPLC. Peptide (4 μmol) was suspended in dimethylformamide, 5% triethylamine, and 4 μmol of hexamethylfluorescein-N-hydroxysuccinimide (Molecular Probes) was added. The reaction was allowed to proceed for 5 min at 4 °C. The products were separated by C18 reverse phase HPLC. The desired peptide, fMBpaFYK-fl was identified by its absorbance and fluorescence spectra (in 10 mM NaCl, pH 10.5), and its mass (m/z = 1340) measured by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-MS) in a α-cyano-4-hydroxycinnamic acid matrix. Samples were stored in amber vials at −20 °C. The syntheses and all manipulations were carried out under dim light.

Preparation of Antifluorescein-Sepharose—Antibodies to fluorescein isothiocyanate were prepared by injecting rabbits with fluorescein isothiocyanate-labeled keyhole limpet hemocyanin, as described previously (16). Serum was precipitated with 50% ammonium sulfate and antibodies were purified on fluorescein isothiocyanate-labeled amine-olexyl-Sepharose. Purified antibodies bound ∼2 mol of fluorescein isothiocyanate-ethanolamine/mol of antibody, as assessed by its ability to quench fluorescein fluorescence. The antibodies were reacted with CNBr-activated Sepharose and cross-linked with 50 μdimethyl pimelimidate (Pierce) to 0.1 μM Na2CO3, pH 10.5, for 16 h. The antifluorescein-Sepharose was washed extensively (>72 h) with phosphate-buffered saline, 1.5 μM NaCl, and 50% ethylene glycol. It was washed quickly (<5 min) with 1% triethylamine, 1.5 μM NaCl, 50% ethylene glycol, and equilibrated in 10 mM HEPES (pH 7.4) and 1% octyl glucoside just prior to use. The antifluorescein-Sepharose bound ∼16 μmol of fMBpaFYK-fl per μl of Sepharose.

FACScan Binding of fMBpaFYK-fl to FPR-expressing Chinese Hamster Ovary (CHO) Cells—FACScan analysis was carried out as described previously by (17). The analysis was carried out in dim light to prevent photolysis of fMBpaFYK-fl.

Photolabeling of fMBpaFYK-fl to FPR and Preparation of CHO Membranes—CHO cells expressing FPR (17) or WT CHO cells were grown in 15-cm tissue culture dishes and treated with 6 μM sodium butyrate 16 h prior to labeling. The cells were washed 3 times with phosphate-buffered saline and incubated with 100 μM fMBpaFYK-fl for 5 min at 4 °C. The tissue culture dishes were loaded directly into a Rayonet RPR-100 UV photoreactor and exposed to UV irradiation for 15 min at 4 °C. EDTA, leupeptin, and IMLF were added to give final concentrations of 1 mM, 1 μg/ml, and 10 μM, respectively. The cells were harvested by scraping and centrifuged at 150,000 × g for 30 min. The cell pellets were sonicated in phosphate-buffered saline, 1 mM EDTA, 1 μg/ml leupeptin, 10 μg/ml IMLF, and 200 μg/ml dithiothreitol (Buffer A), washed twice with 10 mM NaCl, 50 mM ethylene glycol, and 1 mM EDTA, 1 μg/ml leupeptin, 10 μM IMLF, and 200 μg/ml dithiothreitol.

HPLC Analysis of Photolabeled FPR—Membranes from CHO cells expressing FPR and wild type control CHO cell membranes were prepared as described above, resuspended in 0.7 ml of Buffer A containing 3% octyl glucoside, sonicated, and centrifuged at 150,000 × g for 30 min. The supernatant was injected onto anion exchange (Vydac 300VHP) and gel filtration (TSK GW3000) HPLC columns connected in series, and the columns were monitored for fluorescence with excitation at 490 nm and emission at 520 nm. 1 ml of 1x NaCl was injected after 60 min elution to remove material that bound to the anion exchange column. The HPLC analysis was carried out using a Hitachi 6200 HPLC system with a F-1050 fluorescence detector.

Digestion of Photolabeled FPR with CNBr and Isolation of Photocross-linked Fragments—Photolabeled membranes from FPR-expressing and WT CHO cells were washed with H2O, the membranes were resuspended in 200 μl of H2O, sonicated, and dissolved by addition of 800 μl of trifluoroacetic acid. 50 μmol of CNBr was added, and the reaction mixtures were incubated for 16 h at 20 °C. An additional 10 μmol of CNBr was added, and the incubation continued for an additional 8 h. The samples were frozen at −80 °C and lyophilized. The samples were resuspended in 10 mM HEPES and 1% octyl glycoside, adjusted to pH 7–8 and centrifuged at 150,000 × g for 30 min. The supernatant was added to 30 μl of rabbit antifluorescein antibodies bound to Sepharose and incubated for 16 h at 4 °C. The Sepharose beads were washed 5 times with H2O, eluted with 1% triethylamine, 40% acetonitrile in water and lyophilized. For trypsinization, the sample was resuspended in 1% octyl glycoside, 10 mM Tris, pH 8.0, and incubated with 1 μg of trypsin (sequencing grade, Boehringer Mannheim) for 16 h at 25 °C. 4 μg of soybean trypsin inhibitor was added, followed by the addition of 30 μl of antifluorescein immunosorbent matrix, and the isolation procedure described above was repeated.

GTPγS Binding to FPR-expressing CHO Cells—Membranes from FPR-expressing CHO cells were harvested, sonicated, washed in Buffer A and resuspended in GTPγS binding buffer. Binding assays were performed in 1 ml of 10 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM Mg2+, 1 μM GDP. Samples containing 30 μg of protein were preincubated with formyl peptide or analog at 30 °C for 10 min, 0.05 μCi of [35S]GTPγS (100 Ci/mmol) was added, and the reaction continued for 6 min. The samples were filtered through BA 85 0.45-μm filters (Schleicher & Schuell); the filters were washed with 5 ml of 10 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM Mg2+ buffer, and the bound [35S]GTPγS was measured by liquid scintillation counting.

RESULTS AND DISCUSSION

The novel synthetic fluorescent photoaffinity analog fMBpaFYK-fl activates the formyl peptide receptor more efficiently than does fMLF. Fig. 1A shows that fMBpaFYK-fl enhanced [35S]GTPγS binding to CHO cell membranes expressing FPR with an EC50 of 3 nM. This EC50 was about 2-fold lower than...
that observed with fMLF (EC_{50} = 6 nM). At saturation, both ligands increased GTPyS binding to a similar extent (~100% increase in binding). Fig. 1B shows FACSscan measurement of the binding of fMBpaFYK-fl to FPR expressed in CHO cells. Analysis of the FACSscan binding data by nonlinear least squares fit of the data to a sigmoidal curve, using Prism 2 (Graph Pad Software, Inc.), indicated that fMBpaFYK-fl bound to FPR in intact cells with a K_d of 37 nM. This photoaffinity analog had a binding affinity similar to that observed with the hexapeptide, formyl-Nle-Leu-Phe-Tyr-Nle-Lys-fluorescein, a similar fluorescent analog of fMLF that had been previously studied (17–19).

UV irradiation of FPR occupied with fMBpaFYK-fl, produced extensive nonreversible binding of the photoactivatable ligand, as shown in Fig. 2. After 10 min of irradiation, greater than 40% (n = 2) of the fMBpaFYK-fl was no longer displaced from FPR by fMLF under conditions that completely displaced fMBpaFYK-fl, which had not been exposed to UV light.

To determine if fMBpaFYK-fl specifically photolabeled FPR, octyl glucoside-solubilized membranes from CHO cells expressing FPR or WT control CHO cells, which had both been exposed to fMBpaFYK-fl and UV irradiated, were injected onto anion exchange and gel filtration HPLC columns connected in series. The columns were monitored for fluorescein fluorescence (excitation 490 nm/emission 520 nm). 1 ml of 1 M NaCl was injected at time = 60 min to remove material that bound to the anion exchange column and subject that material to size separation by gel filtration. Fig. 3 shows a large peak of fluorescein labeled protein from FPR-expressing CHO cells that was not retained by the anion exchange column (consistent with an isoelectric point of 9.4 for FPR calculated from its amino acid sequence). This peak was found to FPR by Western blot (not shown) and was completely absent in the WT controls, indicating that the fMBpaFYK-fl was specifically labeling FPR. The area under the FPR peak in Fig. 3 represented 60–70% of the eluted fluorescein fluorescence and indicated that most of the photolabeled material was in FPR.

To determine the site of photocross-linking between fMBpaFYK-fl and FPR, photolabeled FPR was cleaved with CNBr and the fMBpaFYK-fl-containing adducts were identified by MALDI mass spectrometry. Fig. 4 shows a model of the transmembrane topology of FPR (17) where the positions of the methionine CNBr cleavage sites are indicated by gray shading. The table inset shows the expected masses of the 11 possible cross-linked adducts between CNBr-digested FPR and benzylphenylalanine-FYK-fl (BpaFYK-fl), the CNBr cleavage product of fMBpaFYK-fl. All of the expected masses of the CNBr cleavage products are sufficiently different that they can be resolved by MALDI-MS, which has a mass accuracy of about 0.1%.

Since the HPLC analysis indicated that most of the photoaffinity label was incorporated into FPR, CNBr digestions were performed directly on CHO cell membranes to maximize the yield of photocross-linked peptides. To obtain sufficient photolabeled FPR for identification of CNBr fragment adduct(s) by MALDI-MS and to show that these adduct(s) were derived from FPR, 20 15- × 1 cm tissue culture plates from WT CHO cells and CHO cells expressing FPR were photolabeled with 100 nM fMBpaFYK-fl. Membranes from an equal number of cells of each type were solubilized in 1 ml of 80% trifluoroacetic acid and cleaved with CNBr.
Fluoresceinated Adducts from Both Wild Type and FPR-expressing CHO Cell Membranes—Fluoresceinated adducts from both wild type and FPR-expressing CHO cell membranes were isolated with antifluorescein immunoaffinity chromatography, as described under “Materials and Methods.” 20 and 8 pmol of cross-linked fluoresceinated peptides were isolated from FPR-expressing and WT CHO cell membranes, respectively, indicating that 60% (i.e. 12 of 20 pmol total) was specifically photocross-linked to FPR, in agreement with the HPLC analysis shown in Fig. 2. The material eluted from the antifluorescein antibodies was analyzed by MALDI-MS (Fig. 5, inset). Mass spectra obtained from WT cells was subtracted from that observed for FPR-expressing cells, and a typical difference spectrum is shown in Fig. 5.

Three major mass peaks were observed at 1182, 1754, and 1913 Da (see Fig. 6B for an expanded view of the difference spectrum). These values corresponded to the expected masses of BpaFYK-fluorescein (1182), BpaFYK-fl cross-linked to the FPR CNBr peptide Val-Arg-Lys-Ala-Hse (1755), and fMBpaFYK-fl (i.e. the uncleaved peptide) cross-linked to the FPR peptide Val-Arg-Lys-Ala-Hse (1913). The mean mass difference between cleaved, uncross-linked agonist peptide and the cross-linked and cleaved peptide was 573.6 Da, which is the mass of the FPR CNBr peptide Val-Arg-Lys-Ala-Hse (FPR residues 83–87). The average mass difference, representing the FPR cross-linking site, was determined from four separate CNBr cleavage reactions of cross-linked FPR, and the same mass peaks at m/z at 1182, 1755, and 1913 were observed in all four CNBr cleavage reactions.

To confirm that the FPR sequence Val-Arg-Lys-Ala-Hse was the fMBpaFYK-fl photocross-linking site, peptides from the antifluorescein eluate were further cleaved with trypsin, purified using antifluorescein antibodies, and subjected to MALDI mass spectrometry. After trypsinization, a major mass peak of 1582 was observed (Fig. 6C) indicating a cross-linked FPR fragment of 401 Da, very similar to the expected mass of Val-Arg-Lys (expected mass 401, FPR residues 83–85). Fig. 6 shows a comparison of the mass spectra of fMBpaFYK-fl cleaved with 10 mM CNBr (Fig. 6A) to the CNBr cleavage product of fMBpaFYK-fl cross-linked to FPR (Fig. 6B) and fMBpaFYK-fl cross-linked to FPR cleaved with both CNBr and trypsin (Fig. 6C). The mass axes of Fig. 6, B and C both show the intermediates of incomplete cleavage of fMBpaFYK-fl by CNBr as also seen in Fig. 6A. These additional cross-linked fragments serve as fingerprints of fMBpaFYK-fl cross-linking and provide additional mass spectral peaks, which are characteristic of the agonist peptide and can thus be used to support the identification of cross-linked peptides.

Three-dimensional Placement of the fMLF Binding Site in FPR—The ability to photoaffinity cross-link FPR with a high affinity analog of fMLF provides an opportunity to experimentally test the intramembrane ligand binding hypothesis for G protein-coupled peptide receptors (17, 18). A three-dimensional model of FPR was generated from a rhodopsin template designed by Herzyk and Hubbard (20), and a space filling model of fMet-Bpa-Phe (fMBpaF) was placed between the seven transmembrane helices (Fig. 7). Two stereo views are shown in Fig. 7, a view from the extracellular space and a transmem-
brane view. fMBpaF was positioned between the helices to
allow maximum contact with residues previously shown to be
important in ligand binding (17). By modeling fMBpaF in an
extended conformation, the Bpa moiety could be placed near
the site of cross-linking while maintaining interactions be-
tween fMBpaF and residues that affect binding. The methio-
nine and phenylalanine side chains of fMBpaF, which are es-
sential for high affinity binding, were positioned deep within
the binding pocket (relative to the extracellular side), whereas
the Bpa side chain was positioned nearer the extracellular
space, so as to be consistent with the cross-linking data. Since
studies with model compounds suggest the carbonyl carbon of
the benzophenone moiety of Bpa generally reacts with another
atom within a distance of approximately 3 Å and the carbon
adjacent to the ϵ amino group of lysine is reported to be one of
more reactive side chains toward photocross-linking with ben-
zophenone (15), the ϵ carbon of Lys-85 was placed within 3 Å of
the carbonyl oxygen of the benzophenone moiety of fMBpaF. In
this position, the amine nitrogen of Lys-85 (II-24) is within 2–3
Å of the methineine carbonyl oxygen on fMBpaF, sufficiently
close to form a hydrogen bond. Alternatively, Lys-85 could ion
pair with Asp-284 and still be close enough to the carbonyl
oxygen of the benzophenone moiety of fMBpaF to photocross-
link effectively. In the present model, neither of the sidechains
of Val-83 or Arg-84 appear to be sufficiently close to the ben-
zophenone carbonyl of fMBpaF to be likely sites of cross-link-
ing, but their backbone moieties might be close enough to
cross-link efficiently. The present data does not distinguish
between cross-linking to the three residues (83–85), and addi-
tional studies will be needed to delineate the exact site(s) of
interaction.

We have previously shown that mutation of FPR Leu-78
(II-17; helix II, residue 17 from the N terminus of the helix) or
Phe-291 (VII-11) to Ala markedly reduced ligand binding (17).
These residues are 2 helical turns toward the cytoplasmic side
of the membrane from Lys-85 (II-24) and Asp-284 (VII-4), re-
spectively. Table I shows the sequences of the residues of hu-
man FPR surrounding Lys-85 (II-24) and Asp-284 (VII-4)
aligned with the analogous residues of rabbit, mouse, gorilla,
and macaque FPRs. Also shown are the homologous sequences
of other closely related chemotactic GPCR’s; lipoxin A4 receptor
(LPXA4R), C5a receptor, interleukin 8 and 8A receptors
(ILA8R and ILA8AR) and a human coreceptor for HIV (CCR5).
These receptors either have Lys at position II-24 and Asp at
position VII-4 (underlined in Table I) or they lack both Lys
and Asp at these positions. The correlation in occurrence of these
two residues suggests that they might interact in the structure,
possibly by forming an ion pair. This ion pair might be similar
to the putative ion pair between Glu-113 (III-3) and Lys-296
(VII-11) in opsin (21) or Asp-103 (III-7) and Lys-331 (VII-4) in
the α1B adrenergic receptor (22). Both of these putative ion
pairs are thought to be important in maintaining these recep-

FIG. 5. MALDI difference spectrum of fMBpaFYK-fl cross-linked to FPR. fMBpaFYK-fl cross-linked to CHO cells expressing FPR or wild
type CHO cells were cleaved with CNBr and isolated as described under “Materials and Methods.” The samples were resuspended in 0.1%
trifluoroacetic acid, 50% acetonitrile, an equal volume of saturated α-cyano-4-hydroxy cinnamic acid in 0.1% trifluoroacetic acid, 50% acetonitrile
was added; the samples were air dried and analyzed by MALDI-MS. The background spectrum seen with wild type CHO cells was subtracted from
the spectra observed with FPR-expressing CHO cells. Three major peaks were observed at 1182, 1754, and 1913 Da, which corresponded to the
expected masses of BpaFYK-fl (1182), BpaFYK-fl-Val-Arg-Lys-Ala-homoserine (1755), and fMBpaFYK-fl (i.e. uncleaved peptide) cross-linked to
Val-Arg-Lys-Ala-homoserine (1913). The position of Val-Arg-Lys-Ala-homoserine in FPR is indicated in Fig. 4. Upper inset, MALDI-MS of
fMBpaFYK-fl cross-linked to CHO cells expressing FPR; lower inset, MALDI-MS of fMBpaFYK-fl cross-linked to WT CHO cells.
tors in their inactive conformation, since mutation of either of the residues involved in the ion pairs results in constitutively active receptors (21, 22). Both human and rabbit FPRs bind fMLF with high affinity and both contain Lys at position II-24 and Asp at position VII-4, whereas mouse FPR, human LXA4R, and human FPRL2 bind fMLF with much lower affinity, and these three receptors all lack both Lys at position II-24 and Asp at position VII-4 (23–25). If, in fact, Lys-85 and Asp-284 do form an ion pair in FPR and ligand binding serves to disrupt such pairing, then Lys-85 might be positioned well to interact with the benzophenone carbonyl as is shown in Fig. 7.

Previous studies with other GPCR have implicated analogous regions of the putative second transmembrane spanning region as being important in ligand binding. Site-directed mutagenesis of the neurokinin-1 and neurokinin-2 receptors identified four residues in the putative second transmembrane helix as important in the binding of substance P and neurokinin A, respectively, to these receptors (26, 27). One of the residues identified as important in ligand binding in the two receptors, Tyr-92 (Tyr-93), is analogous to Lys-85 in FPR, is the most likely ligand cross-linking site in FPR in our experiments. Another residue important for ligand binding in the two receptors, Asn-85 (Asn-86), is analogous to Leu-78 in FPR. The neuropeptide Y receptor also contains a Tyr in an analogous position to Lys-85 in FPR. Mutation of this residue to Phe substantially reduced the binding of neuropeptide Y to the neuropeptide Y receptor suggesting that the OH group of Tyr makes an important hydrogen bond with ligand (28). The lutropin/chorionic gonadotropin receptor has also been analyzed at the analogous position in helix II (29). In the lutropin/chorionic gonadotropin receptor case, mutation of Asp in this position to Lys did not alter ligand affinity, but completely blocked ligand activation of adenylate cyclase. A complementary mutation of a Lys residue in the normal peptide ligand (Lys → Asp) resulted in a ligand which would activate the Asp → Lys receptor mutant but not the WT or the Asp → Ala receptor mutant. This was strong evidence that a complementary interaction between ligand and the receptor residue in the position analogous to Lys-85 in FPR was essential for receptor activation.

Evidence for the location of the ligand binding sites of many

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**Fig. 6.** MALDI-MS analysis of CNBr and tryptic digests of fMBpaFYK-fl and its cross-linked adducts to FPR. A, fMBpaFYK-fl was cleaved with 10 mM CNBr in 80% trifluoroacetic acid, and 16 pmol were analyzed as described in Fig. 5. B, MALDI spectrum from Fig. 5 on an expanded scale. C, fMBpaFYK-fl was cross-linked to FPR-expressing CHO cells, cleaved with CNBr, and subsequently cleaved with trypsin, and analyzed by MALDI as described under “Materials and Methods” and the legend to Fig. 5. The x axes of panels B and C were offset 573 (mass of Val-Arg-Lys-Ala-Hse) and 401 (mass of Val-Arg-Lys) Da, respectively, to align with the CNBr cleavage intermediates of fMBpaFYK-fl seen in panel A.
GPCR has been provided using site-directed mutagenesis. However, very few of these sites have been analyzed directly by ligand cross-linking. An important residue in the binding site of the β adrenergic receptor was identified as Trp-330 (VII-8) by photoaffinity labeling with iodocyanopindolol-diazirine (30). A chemically reactive muscarinic agonist cross-linked to the m1 muscarinic receptor at residue Asp-105 (III-7) (31). Both of these sites, as is the case for the ligand binding site of FPR, map to transmembrane regions near the extracellular face of these receptors. The ligand binding sites on FPR, the β adrenergic receptor, and the muscarinic receptor, all map to a region very similar to that found for the retinal binding site of rhodopsin (32–43). Therefore, we conclude that the binding sites for many different types of GPCR ligands appear to reside in a very similar region of GPCR within the transmembrane region near the extracellular face of the membrane.

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TABLE I
Alignment of sequences of representative chemotactic receptors near the extracellular interfaces of helix II and helix VII

| Helix II | Helix VII | Reference |
|---------|-----------|-----------|
| Human FPR | L1PFVFVRKAM26 | IAVDVTST7 | 45 |
| Rabbit FPR | L1PFVFVTEAL26 | IAVDVTST7 | 24 |
| Gorilla FPR | L1PFVFVREAM26 | IAVDVTST7 | 46 |
| Macaque FPR | L1PFMFVEM26 | IAVDVTST7 | 46 |
| Mouse FPR | L1PFYIAASM26 | TALKITST7 | 25 |
| Human LPXαR | L1PFIVSMAM26 | I1VNPT5ST7 | 47, 48 |
| Mouse LPXαR | L1PFIVSIM26 | R1VNPT5ST7 | 49 |
| Human FPR-L2R | L1PFVMVSAM26 | V1INPT5ST7 | 23 |
| Human C3AR | L1PFLYTVQ26 | T1MPIT5T7 | 50 |
| Human C4AR | L1PFLSRLAL26 | S1VHVT5T7 | 51 |
| Human IL8R | L1PILWAVSV26 | R1GALQTR52 | 52 |
| Human IL8AR | L1PILWAVSV26 | R1GALQTR52 | 53 |
| Human CCR5 | L1PVIWAHQA26 | Q1AMQVTET54 | 54 |

A chemically reactive muscarinic agonist cross-linked to the m1 muscarinic receptor at residue Asp-105 (III-7) (31). Both of these sites, as is the case for the ligand binding site of FPR, map to transmembrane domains near the extracellular face of these receptors. The ligand binding sites on FPR, the β adrenergic receptor, and the muscarinic receptor, all map to a region very similar to that found for the retinal binding site of rhodopsin (32–43). Therefore, we conclude that the binding sites for many different types of GPCR ligands appear to reside in a very similar region of GPCR within the transmembrane region near the extracellular face of the membrane.

Fig. 7. A three-dimensional model of FPR. Upper panel, Stereo view of FPR model with bound fMBpaF as viewed from the extracellular space. Lower panel, stereo transmembrane view of the model with helices V–VII in the foreground. Helices are colored: I, blue; II, magenta; III, aqua; IV, yellow; V, orange-red; VI, light blue; and VII, orange. The transmembrane segments of FPR were selected based on the multiple sequence alignment of G protein-coupled receptors (GPCRs) by Baldwin (44), except that helix II was assigned to residues 61–87 to be consistent with previous site-directed mutagenesis of FPR (17) and the photocross-linking data presented in this paper. The starting model was generated using the automated rule-based method described by Herzyk and Hubbard (20) by submitting the FPR helix segments I–VII to their GPCR modeling site (http://Expasy.heuge.ch/CAI-bin/Promod-GPCR.pl). Rhodopsin, which has a high sequence homology to FPR, was used as the template. A space filling model of fMBpaF (gray) was placed between the seven transmembrane helices so as to be consistent with evidence from site-specific mutagenesis (17). The plane of the phenyl ring of the peptide ligand was positioned parallel to the long axes of the helices to allow fMBpaF to fit between the helices. Adjustments were made to the model based on our photocross-linking data as follows: Lys-85 (K85) was positioned so that the ε amino group was within hydrogen bonding distance of the methionine carbonyl of fMBpaF, and the benzophenone moiety of fMBpaF was rotated at the β carbon of benzoylphenylalanine to position it within 0.3 nm of the side chain of Lys-85. The sidechains of FPR Val-83 (V83), Arg-84 (R84), Lys-85 (K85), and Asp-284 (D284) are represented as CPK space filling models with carbon green, hydrogen white, nitrogen blue, and oxygen red. An asterisk is placed close to the benzophenone carbonyl oxygen photocross-linking site, which is colored red.
