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Expression and Purification of Recombinant Human *N*-Formyl-L-leucyl-L-phenylalanine (FMLP) Receptor

GENERATION OF POLYCLONAL ANTIBODY AGAINST FMLP RECEPTOR

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ABSTRACT. The recombinant formyl peptide receptor has been successfully expressed and purified, utilizing an *Escherichia coli* expression system. Purification of formyl peptide receptor was performed using gel filtration chromatography and affinity chromatography, and the purified protein migrated at an apparent molecular mass of 36,000 Da. The purified recombinant receptor retained functional activity as determined by a ligand binding assay. The yield of the recombinant purified receptor was ~1 mg/2 L of culture, and the binding activity was determined to be ~8 nM, which suggests the conclusion that glycosylation does not affect significantly ligand binding of the *N*-formyl-L-leucyl-L-phenylalanine (FMLP) receptor molecule. The recombinant receptor protein yield was found to be significantly higher than that obtained from neutrophils. The purified recombinant receptor was then utilized to generate antibody against the same. The reaction of the antibody against recombinant formylpeptide receptor and against native formylpeptide receptor on neutrophils was confirmed by western blot analysis and flow cytometric analysis, respectively. The antibody was also used successfully to detect recombinant formylpeptide receptor expression on transfected 293 cells. These results describe for the first time the expression, purification, and characterization of recombinant FMLP receptor with ligand binding activity and the generation of polyclonal antibody against the same. This work also provides a foundation for future biophysical studies of the FMLP receptor molecule, which have not been possible until now. BIOCHEM PHARMACOL **54**;3:381–390, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. FMLP; receptor; neutrophil; purification; recombinant; antibody

Neutrophils migrate from the blood to sites of inflammation in response to locally produced chemoattractants that activate specific cell surface receptors. FMLP§, a bacterial metabolic product, is a potent chemoattractant for neutrophils [1–4]. This peptide, by attaching itself to specific plasma membrane receptors, elicits an array of cellular responses in addition to chemotaxis [5]. These functional responses appear to be manifested through a receptor-mediated transduction mechanism involving the activation of phospholipase C and specific guanine nucleotide binding protein (G_i) [6]. The FMLP receptor is unique among chemoattractant receptors, because, unlike the receptors for C5a or interleukin-8 (IL-8), which detect signals from "within," it recognizes signals from the outside, such as bacterial products. Thus, this receptor plays a major, if not unique, role in polymorphonuclear neutrophil (PMN) bacterial recognition and response. Although other chemoattractants participate in PMN recruitment and activation, the FMLP receptor represents the ultimate direct link between the responding neutrophil and the invading bacteria.

The FMLP receptor on human neutrophils has been reported to consist of multiple components, the major species being a glycoprotein of 55,000–70,000 Da [7, 8]. Deglycosylation with endoglycosidase F leaves a core peptide of ~33,000 Da, which is still able to bind the ligand [9]. Isolation of a cDNA that codes for the human *N*-formylpeptide receptor has been reported [10]. The amino acid sequence deduced from this clone suggests that the FMLP receptor exhibits the topological motif characteristic of other G-protein coupled receptors, which consists of seven hydrophobic (and, potentially, α helical) segments that span the lipid bilayer [11]. Using peptide analogs to different domains of the receptor, Radel *et al.* [12, 13] have shown that charged residues in the first extracellular loop play a critical role in ligand binding. To confirm the finding, we constructed a deletion mutant (deletion of the first 256 bases) that, as expected, did not show ligand binding activity [14]. Other studies using chimeric molecules of FMLP and IL-8 receptors further confirmed the finding [15].

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§ *Abbreviations:* EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide · HCL; FITC, fluoroscein isothiocyanate; FMLP, *N*-formyl-L-leucyl-L-phenylalanine; FMLPK, *N*-formyl-L-leucyl-L-phenylalanine-lysine; FPR, FMLP receptor; PAL, photoaffinity ligand; PMSF, phenylmethylsulfonyl fluoride; TLCK, *n*-tosyl-L-lysine chloromethyl ketone.

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Although the studies described above have provided some information on the receptor molecule, the overall structural and functional analysis of ligand–receptor interaction needs further elucidation. The cloning of the FPR cDNA helped to deduce the primary structure of FPR [10]. Further, some insights into the ligand binding and G-protein binding domains of FPR have been obtained by biochemical, biophysical, and molecular genetic analysis [16–18]. However, details of the FPR structural–functional relationship in respect to ligand binding and G protein binding are far from being achieved. This is due, in part, to the low abundance of purified receptor protein available from neutrophils (0.039 to 0.062 nmol/10^9 cells) [19, 20], which, in turn, makes detailed structural and functional characterizations difficult. In our study, we explored the possibility of expressing FPR in a prokaryotic expression system. Our goal was to prepare and purify quantities of the FMLP receptor that would allow further biochemical and biophysical studies. Since glycosylation in FPR does not play a role in ligand binding [9], the lack of post-translational modification in bacteria was not a concern in our approach. In this paper, we describe the successful expression and purification of human FMLP receptors with ligand binding activity in an *Escherichia coli* expression system. This product may allow studies of purified receptor alone and in re-constitution with other purified signaling components, which, in turn, may permit analysis of receptor-activated cell mechanisms under more defined and controllable conditions.

As part of this study, we also wanted to generate antibodies against the FMLP receptor. The unavailability of antibody against the entire receptor molecule has been one of the serious obstacles in FPR characterization. This has been due primarily to the low amount of FPR available from neutrophils. In addition, using the native receptor molecule as an antigen, because of its heavy glycosylation, may result in the major fraction of the antibodies being directed against sugar moieties rather than the polypeptide chain. Although some investigators have reported the generation of antibodies against particular domains of FPR or modified FPR [15, 21], their uses are found to be limited to only selective assay procedures. For example, antibodies generated against an intracellular domain of FPR may not be suited for detecting FPR surface expression in a mammalian system. Thus, the ideal candidate for generating the polyclonal antibody is the full length non-glycosylated receptor protein that can be obtained from a prokaryotic expression system. In this paper, we document successful generation and functional analysis of polyclonal antibodies, utilizing the purified FPR.

MATERIALS AND METHODS

Cloning and Expression

Cloning and expression of the FMLP receptor in a prokaryotic expression system were performed as described before [14]. In short, the receptor coding DNA was cloned into the expression vector pET-11a (Novagen). The recombinant plasmids were transferred into host cells [BL21(DE3)], and recombinant protein expression was induced by isopropyl-β-D-thiogalactopyranoside (IPTG) (0.1 mM). For mammalian expression, the receptor coding DNA was cloned into an *Eco*RI/*Bam*HI site of the expression vector pSG5 (Stratagene, La Jolla, CA). FPR clones were confirmed by dideoxy sequencing. Human embryonic kidney 293 cells (American Type Culture Collection No. CRL 1573) were used for the transfection procedure. Cells were maintained in DMEM/F12 (50:50) supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY) and antibiotic penicillin/streptomycin. Calcium phosphate-mediated transfection of cells was carried out as per standardized protocol [22] using 8–9 μg of purified recombinant plasmid (Qiagen, Chatsworth, CA). 293 Cells were transfected with recombinant pSG5 and then were cultured for 48 hr. The expression of FPR on 293 cells was determined by flow cytometry using anti-FPR antibodies and FITC-FMLPK (Molecular Probes Inc., Eugene, OR).

Protein Extraction

The cell pellet from *E. coli* cells expressing the recombinant protein was disrupted in buffer containing 2 mM EDTA, 1 mM PMSF, 2 mM TLCK · HCl and lysed by seven cycles of 1-min sonication treatment in an ice bath (Vibra Cell model VC 250; Sonic & Materials Inc., Danbury, CT). Then the disrupted pellet containing inclusion bodies was centrifuged at 10,000 *g* for 30 min. The resultant pellet was subjected to a sucrose shock procedure as follows. The pellet was suspended in 50 mM Tris (pH 8) containing 1 M sucrose and was incubated for 12 hr at 4° with continuous stirring. The mixture was centrifuged at 50,000 *g* for 30 min, and the residual pellet was suspended in low concentration digitonin buffer (0.1% digitonin in 50 mM Tris, 100 mM NaCl, 5 mM EDTA, 2 mg/mL leupeptin, 1 mM PMSF, 2 mM TLCK · HCl and 10 mg/mL benzamidine, pH 7.2). The suspension was stirred for 30 min at 4° and then was centrifuged at 50,000 *g* for 20 min. The supernatant was stored, and the residual pellet was suspended in high concentration digitonin buffer (1% digitonin in 50 mM Tris, 100 mM NaCl, 5 mM EDTA, 2 mg/mL leupeptin, 1 mM PMSF, 2 mM TLCK · HCl, 10 mg/mL benzamidine, 2.5 mg/mL leupeptin, 2.5 mg/mL aprotinin, pH 7.2). After a second 30-min stirring at 4°, the suspension was centrifuged at 100,000 *g* for 30 min. The pellet and the supernatant were both saved for further analysis.

The modification used in the extraction procedure of recombinant FPR as opposed to our previous protocol [14] was the use of a differential digitonin solubilization technique [23]. Use of other detergents as extracting reagent, e.g. CHAPS, Triton X-100, or sodium deoxycholate, was found either inefficient as per the amount of receptor protein extracted or unable to maintain the functional integrity of the receptor protein. We found digitonin to be ideal in extracting recombinant FPR protein.

Purification Method

SEPHAROSE CL-6B CHROMATOGRAPHY. The column was pre-equilibrated at 4° using one column volume of 500 mM NaCl, 50 mM Tris, 0.5% digitonin, and 2 mM EDTA, pH 7.2, followed by one column volume of 100 mM NaCl, 10 mM Tris, 0.05% digitonin, and 2 mM EDTA, pH 7.2. The high concentration digitonin extract was loaded at 4° onto a 200-mL column of Sepharose CL-6B at 60–70 mL/hr. Gel filtration chromatography of the crude preparation was performed using one column volume of 100 mM NaCl, 10 mM Tris, 0.05% digitonin, and 2 mM EDTA, pH 7.2 (chromatography buffer).

SEPHADEX G-75 CHROMATOGRAPHY. The fractions from the Sepharose CL-6B column were loaded at 4° onto a 100-mL column of Sephadex G-75 at 50–60 mL/hr. The column was equilibrated the same way as for the Sepharose CL-6B column, and gel filtration chromatography was performed using the chromatography buffer.

AFFINITY CHROMATOGRAPHY. AH-Sepharose 4B (4 mL) was washed sequentially with 200 mL of 0.5 M NaCl and 300 mL of distilled water. The washed gel was incubated for 6 hr at 37° with 12 mmol of fMet-Leu-Phe dissolved in 40 mL of DMSO and 40 mg of EDC (Pierce, Rockford, IL) in 10 mL of distilled water (pH 4.5). A second equal amount of EDC was added, and the mixture was incubated at room temperature for 10 hr. The fMet-Leu-Phe-Sepharose was washed with 200 mL of 0.5 M NaCl and 100 mL of distilled water and stored at 4°. An fMet-Leu-Phe-Sepharose column of 1.5 mL bed volume was washed with 500 mM NaCl, 50 mM Tris, 0.5% digitonin, and 2 mM EDTA, pH 7.2, followed by one column volume of chromatography buffer. The Sepharose CL-6B column fractions that assayed positive for the FMLP binding activity (see below) were then loaded onto the column at room temperature. After the column was washed with 15 mL of chromatography buffer at 4°, the receptor protein molecules were eluted at room temperature with 4 mL of chromatography buffer containing 20 mg of fMet-Leu-Phe dissolved in 200 mL of DMSO. The eluate was dialyzed against 2 L of chromatography buffer (pH 8.0) for 48 hr at 4°. The eluate was then concentrated to 1 mL using Centricon-30 (30,000 mol. wt cut-off) (Amicon, Beverly, MA) concentrators.

Receptor Binding Assay

QUALITATIVE BINDING ASSAY. The assay was carried out using a PAL as described before [14]. Briefly, 30 μL of receptor preparations (ranging between 10 and 30 μg) was incubated with 1–2 nmol of [^{125}I]PAL (~ 1.0×10^6 cpm) at room temperature for 15 min with reaction pH ranging between 7.0 and 8.0. Covalent bonding of the label was achieved by photolysis under long-wave UV light for 5 min. The reaction mixtures were then analyzed by SDS-PAGE/autoradiography. Specific receptor binding was determined by competing the radiolabeled PAL with unlabeled FMLP.

QUANTITATIVE BINDING ASSAY. The PAL was incubated with the receptor protein preparation for 15 min at room temperature at 4°. After incubation, the PAL was cross-linked with the bound receptor protein molecules by long-wave UV exposure for 5 min. After UV exposure, 0.1% γ-globulin was added to the sample as a carrier protein, and the mixture was incubated for 5–10 min at room temperature with occasional vortexing. To separate the unbound ligand from the bound ligand, 10% PEG (polyethylene glycol 8000) (Sigma, St. Louis, MO) was added to the reaction mixture, mixed thoroughly by mild vortexing, and kept on ice for 30 min. The ligand bound fraction was then precipitated by centrifugation at 10,000 *g* for 20 min. The precipitated pellet was washed twice with PBS (pH 7.5), and subsequently the radioactivity of the pellet was quantified in a γ-counter.

Preparation of Polyclonal Antibodies (anti-FPR)

Female New Zealand white rabbits (5–6 lb) were immunized at multiple intradermal sites with 200 μg of purified recombinant FMLP receptor protein in 0.5 mL of isotonic saline emulsified with an equal volume of Freund's complete adjuvant. Animals were boosted at weekly intervals with 30 μg of the antigen emulsified with Freund's incomplete adjuvant. Six weeks later, the rabbits were bled and the sera were tested for antibody by immunoblotting. Pre-immune sera were also obtained and analyzed in a parallel manner.

Isolation of Antibody

IgG isolation from anti-FPR sera was carried out using an ammonium sulfate precipitation protocol [24]. Anti-FPR antibodies precipitated (40% saturated ammonium sulfate) from 1 mL of anti-FPR sera were redissolved in the same volume of 1× PBS (pH 7.5) and dialyzed overnight against the same.

Isolation of Human Neutrophils

Normal human neutrophils were isolated from fresh buffy coats according to the method described by De Nardin *et al.* [25]. The neutrophil yield from one buffy coat (~ 400 mL) was ~2×10^9 cells with a viability >96% as assessed by the exclusion of trypan blue stain.

Flow Cytometry

Neutrophils were suspended in 500 mL of PBS (Ca^{2+} and Mg^{2+} free) at a concentration of 4×10^6/mL. Anti-FPR antiserum was added to the neutrophils at a concentration of 1:500 and incubated in a shaker at 4° for 2 hr. After incubation, the cells were washed twice in PBS, resus-

pended in 500 mL of PBS, and a second antibody (goat-anti-rabbit-FITC) was added at a concentration of 1:250. The cells were incubated at 4° for 1 hr, washed twice in PBS, and then resuspended in 500 mL of fixative solution (1% formaldehyde, 2% bovine serum albumin, in PBS). The expression on 293 cells was determined in a similar manner except that in this case anti-FPR antibodies were used at concentrations of 1:100 and 1:200. Cells incubated with FITC-fMet-Leu-Phe (10^{-6} M) (Molecular Probes Inc.) instead of anti-FRP sera, as well as cells incubated with second antibody alone, served as controls. Pre-immune serum was used to determine non-specific binding. Analysis was performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) using the Cell Quest Program (Becton Dickinson). Contaminants and dead cells were excluded with a gate on forward and side scatter.

Amino Acid Analysis and N-Terminal Amino Acid Sequencing

These procedures were carried out as described before [14].

Analytical Procedures

Protein concentration was determined by the method of Bradford [26] using bovine serum albumin as the standard. SDS–PAGE was performed in a minigel system (Mini-Protean II cell; Bio-Rad, Hercules, CA), using a 1-mm spacer. Both 10% and 7.5% running gels were performed according to the method described by Laemmli [27]. Proteins separated by SDS–PAGE were transferred to nitrocellulose membranes in 25 mM Tris-glycine buffer (pH 8.3) for the western blotting procedure.

RESULTS

Cloning of the FPR

The cloning of FPR coding DNA into the prokaryotic expression vector pET-11a and eukaryotic expression vector pSG5 was confirmed by restriction enzyme digestion and subsequent agarose gel electrophoresis analysis. Nucleotide sequencing revealed the presence of an open reading frame of 1050 bases, which was in accord with the published sequence by Boulay *et al.* [10].

Expression and Purification of the Recombinant FMLP Receptor

As the initial step of purification, crude recombinant receptor preparation obtained from inclusion bodies and solubilized in digitonin was chromatographed using gel filtration columns. Application of the crude preparation to a Sepharose CL-6B column, pre-equilibrated with buffer (mentioned in Materials and Methods), resulted in the elution of the receptor protein fractions in two-thirds of the bed volume. Protein concentration of the column fractions was assessed by the Bradford assay [26]. Most of the receptor

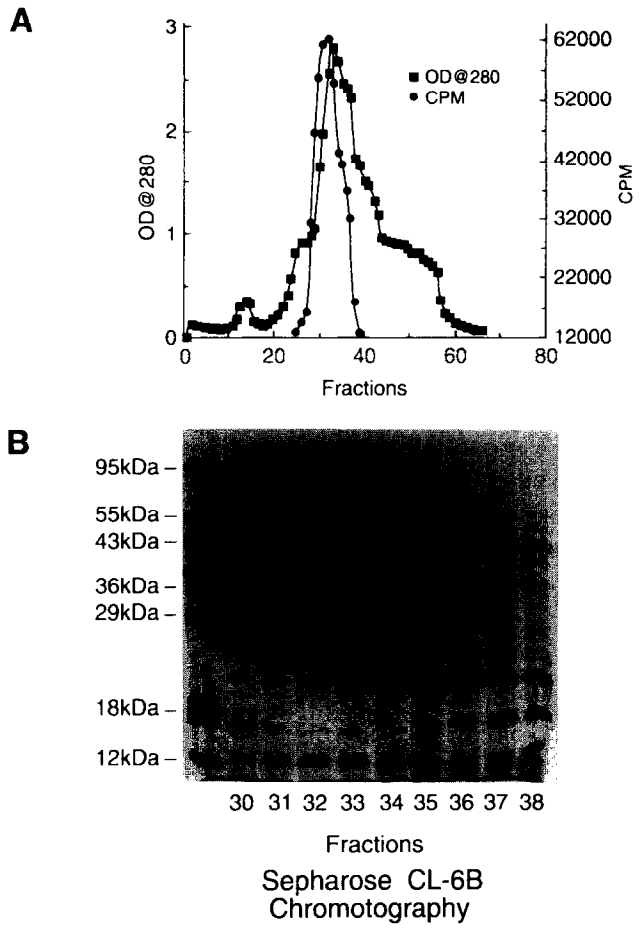

FIG. 1. Sepharose CL-6B column chromatography of crude rFPR. (A) Chromatograph showing protein concentration and the ligand binding activity of eluted fractions, measured as described in Materials and Methods. (B) Eluted fractions analyzed by SDS–PAGE under reducing conditions on a 10% polyacrylamide gel stained with 0.1% Coomassie blue. The major protein band revealed showed a molecular mass of ~36,000. Molecular standard markers are indicated on the left.

binding activity was detected in the eluted fractions corresponding to the early half of the major protein peak (fractions 29–36) (Fig. 1A). The corresponding SDS–PAGE profile of the fractions revealed a major protein band at an M_r of ~36,000 (Fig. 1B), which is a little higher than the molecular mass reported for the deglycosylated native receptor (M_r = 33,000) [9]. The difference in the molecular mass between the recombinant FPR and the deglycosylated FPR may be due to the association of digitonin to the recombinant protein as occurred during the extraction procedure or due to anomalous behavior of the protein molecule itself [28]. Protein yield at this step was around 45% of the total applied protein (Table 1). The ligand binding activity of the eluted receptor fractions (fractions 29–36) after the initial step was found to be ~10-fold as compared with the initial crude receptor protein preparation applied (Table 1). Fractions from the Sepharose CL-6B column that exhibited binding activity were pooled together, concentrated using Centriprep-30 (Amicon), and

TABLE 1. Purification of rFPR

	Protein (mg) (approx.)	Overall yield (%)	Specific activity (fmol/mg protein)	X-fold purif. overall (approx.)
Crude digitonin extract	102.9	100	0.0752	1
Sepharose CL-6B column eluate	46.0	45	0.7630	10
Sephadex G-75 column eluate	23.0	23	4.5870	60
FMLP-affinity eluate	0.879	1	510.25	6700

Analysis of representative purification of rFPR from *E. coli* expression system according to the procedures detailed under Materials and Methods. The yield and fold-purity of the rFPR as per successive steps of purification are shown.

loaded onto the Sephadex G-75 column (equilibrated with chromatography buffer). Fractions were eluted from the column in one-third bed volume, and the major binding activity was detected on protein peak fractions 4–20 (Fig. 2A). SDS–PAGE and subsequent silver staining of fractions (3–11) demonstrated the major protein band in this peak to be ~36 kDa (Fig. 2B). The protein yield at this step was around 23% overall with the purification being ~60-fold (determined as previously stated) (Table 1). Sephadex G-75 column fractions (4–20) exhibiting binding activity were pooled together and applied onto the FMLP-affinity column. A subsequent high salt wash of the column after loading resulted in removal of 87% of the total protein applied. Bound receptor was then eluted with excess ligand FMLP (12 mM) containing chromatography buffer (see Materials and Methods), and the whole affinity procedure was repeated again. Figure 3A shows the results of affinity elution chromatography. The affinity-column-purified FPR showed a protein band of ~36,000 Da as determined by SDS–PAGE/silver staining (Fig. 3B). The binding activity of the affinity-purified FPR was determined by qualitative ligand binding assay (described above), as shown in Fig. 4. Lane 1 shows the binding of the ligand to human neutrophil membrane extracts (used as the positive control). As expected, this resulted in the labeling of a band at ~66 kDa, which is the size of the FMLP receptor from human neutrophils [7]. Lane 2 (negative control) contained extracts from the host bearing the plasmid alone and showed no specific binding to the PAL. Lane 3 shows the binding of the photoaffinity ligand to the affinity column eluate, which resulted in a labeled band of ~36 kDa. The specificity of binding was determined as before [14] by competing PAL with excess unlabeled FMLP, in the above-mentioned assay system, resulting in complete binding inhibition (data not shown). The silver staining (Fig. 3B) also revealed minor bands at a higher size than that of FPR. These protein bands failed to show any ligand binding activity; however, the presence of inactive FPR aggregates cannot be ruled out. The overall yield of FPR was found to be about 800–900 μg. The complete purification resulted in an overall 5% protein recovery with a 6700-fold purification (Table 1). Thus, the recombinant FPR was purified from a prokaryotic expression system with retention of ligand binding capacity.

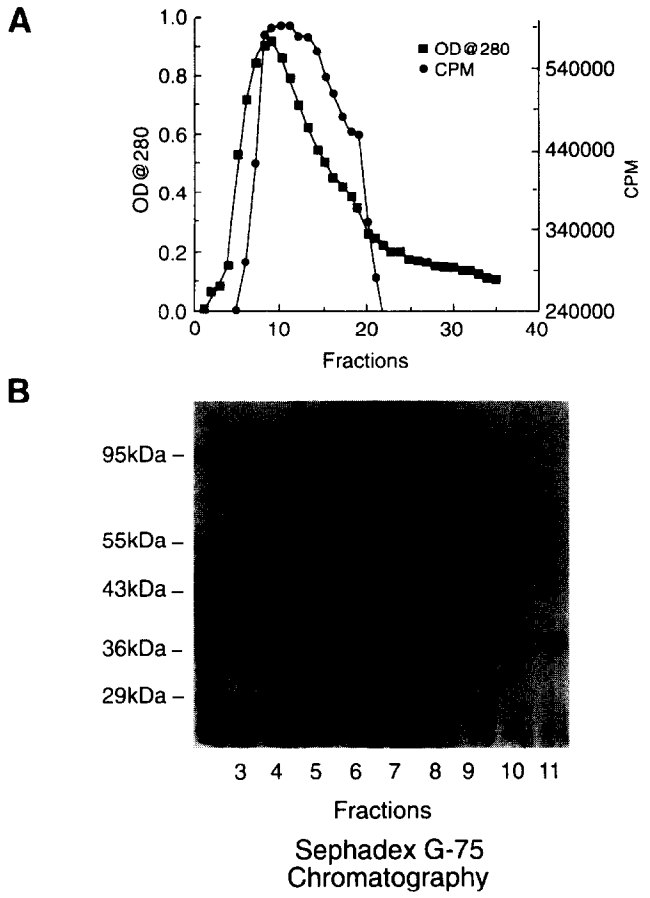

FIG. 2. Sephadex G-75 column chromatography of rFPR containing fractions eluted from Sepharose CL-6B. (A) Chromatograph demonstrating protein concentration and the ligand binding activity of eluted fractions, measured as described in Materials and Methods. (B) Eluted fractions analyzed by SDS–PAGE under reducing conditions on a 10% polyacrylamide gel stained with 0.1% Coomassie blue. The major protein band revealed showed a molecular mass of ~36,000. Molecular standard markers are indicated on the left.

Sequence Analysis

To determine the identity of the purified FPR, amino acid composition analysis was carried out, which was found to be comparable to that reported for the human FMLP receptor [20]. Formylation at the N-terminal of the FPR poses a problem in direct N-terminal amino acid sequencing of FPR. As an alternative approach, N-terminal amino acid

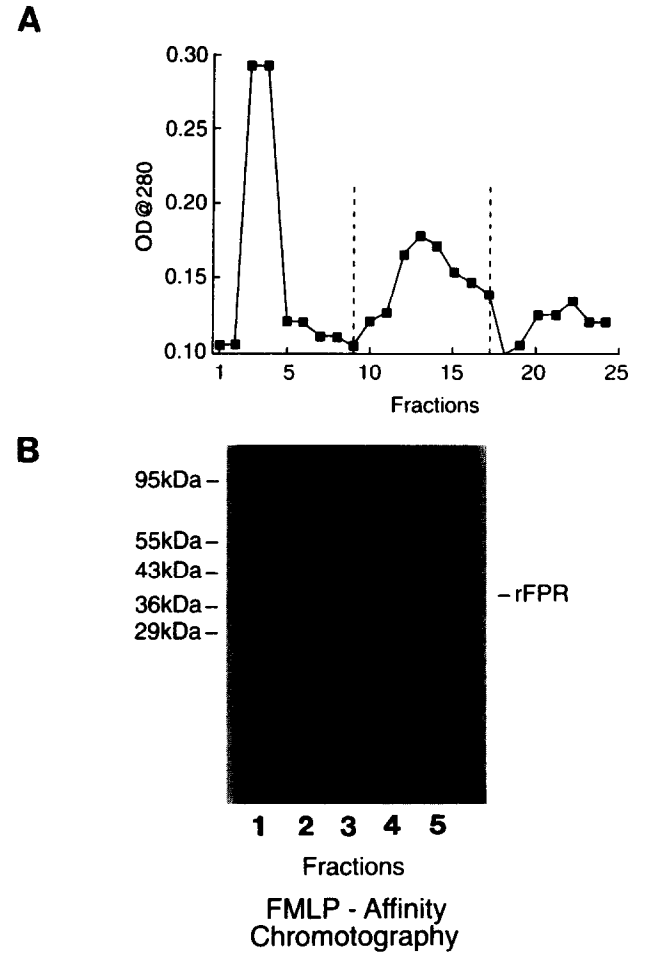

FIG. 3. Affinity purification of rFPR following gel filtration column chromatography. (A) Chromatograph demonstrating the protein concentration of affinity-column-eluted fractions measured as described in Materials and Methods. The fractions within the dotted lines were obtained following elution with chromatography buffer containing FMLP ligand (12 mM). Fractions 1–9 were obtained during wash with chromatography buffer. (B) Silver-stained 10% SDS–PAGE analysis of pooled fraction eluted with excess FMLP ligand (12 mM) containing chromatography buffer. Lane 1, blank; Lane 2, molecular standard markers; Lane 3, blank; Lane 4, the major protein band revealed showed a molecular mass of ~36,000 in the eluted fraction; and Lane 5, blank.

sequencing of fragments was performed following partial trypsin digestion of FPR. Sequencing of the first 10 amino acids of three major fragments of partial tryptic digests of purified FPR revealed sequences corresponding to the sequence of the FPR as published in the literature [10].

Binding Affinity Analysis

The purified recombinant FPR was subjected to a quantitative ligand binding assay technique as described in Materials and Methods. This resulted in the saturable binding curve as shown in Fig. 5. Binding affinity was determined by Scatchard analysis (Fig. 6), and found to be 8 ± 0.8 nM (K_d values ± SD were calculated from the results of three

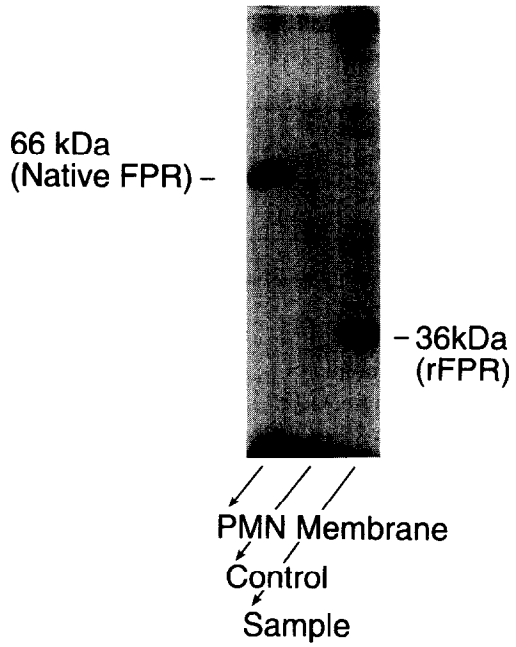

FIG. 4. Qualitative binding analysis of affinity-purified rFPR. The fractions (30 μg each) were analyzed as described in Materials and Methods. Binding analysis of extracts from neutrophil membranes served as positive control. Control denotes extracts from *E. coli* cells containing plasmid (pET-11a) alone. Sample denotes affinity-purified extracts from *E. coli* cells bearing recombinant pET-11a (full-length receptor DNA as the insert).

experiments). As expected, a single binding site was observed in the Scatchard analysis. The binding affinity was similar to that reported previously, i.e. 3 ± 2 nM for tunicamycin-induced deglycosylated native FPR [29].

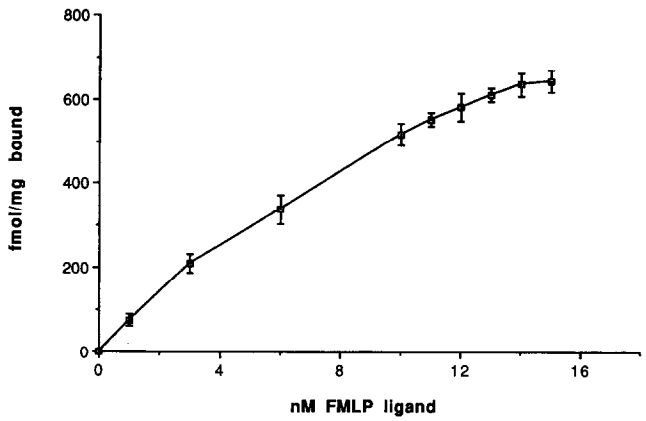

FIG. 5. Quantitative binding analysis of affinity-purified rFPR. Specific binding to affinity-purified rFPR was analyzed as described in Materials and Methods. Non-specific binding was determined using affinity-column-eluted fractions containing no major protein band at molecular mass of ~36,000. The above presented data are a representation of three separate experiments. Data points are means ± SD.

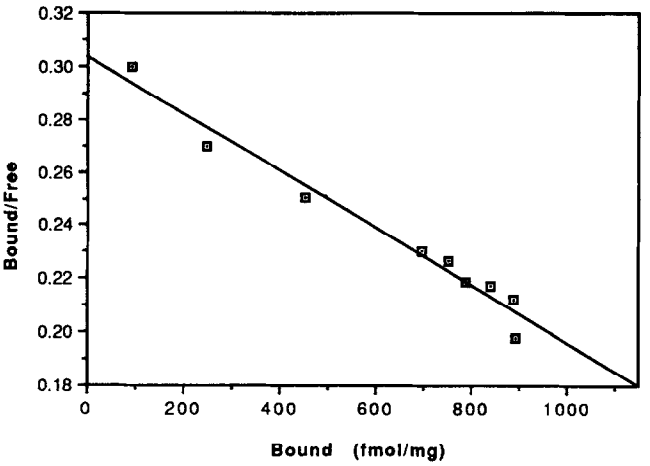

FIG. 6. Scatchard plot analysis of the data obtained from quantitative ligand binding of affinity-purified rFPR ($y = 0.30323 - 1.0712^{e^{-4x}}$; $R^2 = 0.969$).

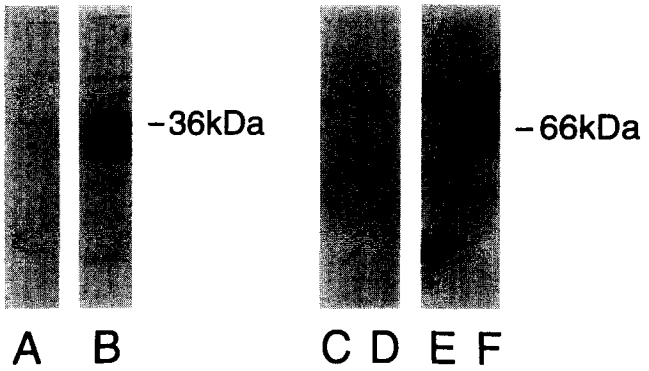

FIG. 7. Western blot analysis using anti-rFPR polyclonal sera, Lanes A and B show the reaction of the crude rFPR preparation against pre-immune sera and immune sera, respectively. Lanes D and F show the reaction of the native membrane preparation from neutrophils against pre-immune sera and immune sera, respectively. Lanes C and E containing 293 epithelial cell extract served as controls.

Analysis of Anti-FPR Antibodies

Polyclonal antibodies (1:1000 dilution in PBS) generated against the purified FPR were tested by western blot analysis. Figure 7 shows the reaction of the antibody against extracts from whole *E. coli* transformed with recombinant plasmid, as well as neutrophil membrane preparations. Approximately 30 μg of protein was loaded in each lane. Pre-immune serum was used as a negative control. The molecular mass (as determined by SDS–PAGE) of the *E. coli* extract component recognized by the antibody was 36 kDa, which corresponds to the size of the recombinant FMLP receptor. The minor bands appearing in the western blot at a lower size may represent proteolytic degradation of the recombinant receptor preparation. The antiserum was also found to react against the native receptor molecule from neutrophil membranes. Specifically, the antisera reacted against a $M_r \sim 66{,}000$ component from the neutrophil membrane preparation, which is the size expected for the FMLP receptor on neutrophils [7] (Fig. 7).

The binding of the anti-FPR sera against whole neutrophils or transfected 293 cells was evaluated by flow cytometric analysis. Again, pre-immune serum was used as a negative control. Figure 8 shows antibody binding to whole neutrophils at a 1:500 dilution in PBS. Similar results were obtained with dilutions ranging from 1:300 to 1:600. Whole neutrophils reacting against FITC-labeled FMLPK were used as a positive control in the flow cytometric assays.

The antisera also demonstrated a difference in reactivity against rFPR-transfected 293 cells as compared with the 293 cell alone. However, this difference was not statistically significant. Pre-immune sera reacting against the transfected and non-transfected 293 cells were used as controls. However, isolated IgG from the crude antisera could specifically react with the expressed FPR in 293 cells. Figure 9 demonstrates the specific binding of anti-FPR IgG (1:200) to transfected 293 cells. Different preparations of purified rabbit IgG molecules (1:200) directed against various *Porphyromonas gingivalis* fimbrial peptides served as negative controls.

DISCUSSION

Activation of the FPR by its ligand initiates a signal transduction cascade that results in many different cellular responses. Elucidation of the critical amino acid residues in the FPR molecule interacting with the ligand has therefore been a major focus of research involving chemotactic receptors. While some progress has been made in identifying regions of the receptor molecule involved in ligand binding [12, 13, 15], more detailed work is needed to fully characterize the ligand binding regions of FPR. There are approximately 6×10^4 receptors on neutrophil membranes [7] and an equal number expressed intracellularly [30]. This represents only 12×10^{-15} g of receptor protein per cell, explaining why purified receptor preparation from isolated neutrophils has so far been elusive. Thus, the unavailability of a sufficient amount of purified FPR has been a major drawback for its structural and functional characterization. Hence, it was the goal of this study to express and purify functional (ligand binding) recombinant FPR in an *E. coli* expression system, and to produce such receptor in sufficient quantities to allow further biochemical and biophysical studies.

This manuscript describes the successful expression and purification of recombinant human FPR in an *E. coli* expression system. This is the first reported purification of one of the receptors belonging to the seven-transmembrane-domain receptor family in a recombinant expression system. It was interesting to note that a significant amount of expression of the rFPR was possible only when we utilized the pET/T7 system. Expression systems used other than pET/T7 had either an extremely low yield or no detectable expression at all. This may be due to the toxicity of the FPR gene product to the host cells, something that has been reported by some investigators [31].

Since in the pET/T7 system the target genes are initially cloned using hosts that do not contain the T7 RNA polymerase gene, plasmid instability does not occur as the

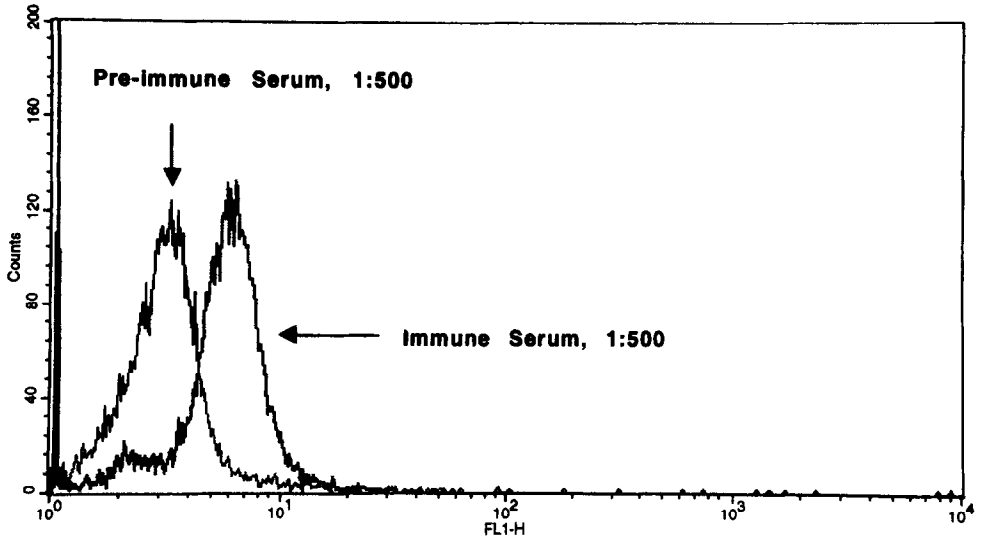

FIG. 8. Flow cytometric analysis of anti-FPR sera activity. Anti-FPR sera activity against whole neutrophils was determined by flow cytometry, plotting cell number versus fluorescent activity.

transcriptional level of the toxic gene is very low or virtually "off" [32]. Furthermore, since we anticipated that the FPR gene product may be toxic, we used carbenicillin in the culture medium instead of ampicillin; this is because carbenicillin prevents the overgrowing of cells lacking plasmid (which happens in plasmid instability due to toxic gene) while ampicillin is destroyed by the β-lactamase produced by the cells in culture [31].

The yields obtained for different recombinant receptors have varied greatly. The yield of r5HT3 receptor in a baculovirus system was in the order of 200 μg/3 L [33], whereas the yield of recombinant porcine M2 muscarinic acetylcholine receptor was 2 mg/1.5 L of CHO cell culture [34]. Therefore, the yield of rFPR obtained in this study is comparable to what has been reported in the literature for other receptors. More importantly, the amount of purified rFPR obtained was much greater than that from whole cells, being at least 10,000 times greater than that obtained from 10^9 neutrophils [20].

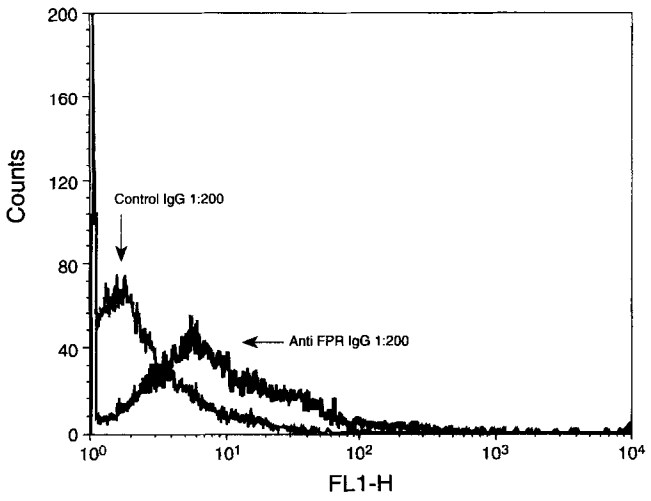

FIG. 9. Analysis of cell surface expression of FPR. Expression was evaluated for transfected 293 cells using anti-FPR IgG by flow cytometry, plotting cell number versus fluorescent activity. Isolated IgG against *P. gingivalis* fimbrial peptides acted as the control.

The binding affinity of the rFPR is somewhat lower than that reported for the FPR isolated from neutrophils. However, this difference is minimal, and may not be functionally significant. Whether this difference is due to the lack of glycosylation on the rFPR remains unclear. Chemical deglycosylation of native FPR has been found not to affect the ligand binding affinity [9]; on the other hand, tunicamycin-mediated deglycosylation has been shown to affect the binding constant [29]. Similar reports on insulin and epidermal growth factor receptor have suggested that deglycosylation can affect ligand binding [35], as tunicamycin used in such studies was found to block receptor activity *in vivo*. Based on these observations, it is possible that glycosylation is necessary to achieve the final folded structure of the receptor but not to maintain it. Thus, chemical deglycosylation does not reduce the ligand binding activity of the native receptor, but blocking of glycosylation at post-translational level may have an effect. This may explain why purified recombinant FPR demonstrated a slight loss of binding activity as compared with the native FPR.

The binding curve in Fig. 5 does show a tendency to shift towards the right when compared with an ideal binding curve. This kind of phenomenon is usually observed in the presence of competitive inhibitors [36]. It may be possible that impurities in the detergent or the detergent itself, even in low concentration, competitively inhibit the binding activity of purified recombinant FPR [37]. The polar interactions between the polar head group of the detergent and neighboring polar groups of the protein might explain such a possibility.

Our results also confirm the previous finding [38] that digitonin was the most appropriate choice of detergent to solubilize FPR. Other detergents such as 0.5% deoxycholate, 0.5% Triton X-100 or 1% CHAPS failed to extract rFPR with ligand binding activity. It is likely that these detergents could not help to maintain the structural integ-

rity of FPR or may have blocked the binding site of the receptor by some biochemical interactions.

In this paper, we also report the generation of polyclonal antibodies to human FMLP receptors. Since these antibodies were generated using a deglycosylated form of the receptor (i.e. the recombinant molecule) as the antigen, one of our concerns was whether the antisera would recognize the native, i.e. glycosylated, receptor. This, indeed, it did, reacting specifically with both human neutrophils, as well as with FPR from neutrophil membranes. The low efficiency of transfection in 293 cells (probably due to the low transfection efficiency of the calcium phosphate method) may explain why purified anti-FPR IgG had to be used at a higher concentration for receptor detection.

The availability of an anti-FPR antibody may provide an additional tool for the study of the receptor beyond the use of labeled ligands. For example, the failure to detect the receptor in a mammalian expression system, using a labeled ligand, may not necessarily indicate that the receptor is absent from the cell surface. It may also indicate a receptor form whose ligand binding ability has been altered. Since the polyclonal antibody reactivity is not dependent on ligand binding, such a reagent can be used as an additional approach to detect the receptor molecules on the cell. This may be true, for example, in site-directed mutagenesis study of FPR ligand binding regions, where a mutated receptor that does not bind the ligand can still be easily detected by an antibody system.

The next step of this study is the structural and functional characterization of FPR using biophysical approaches. Crystallization of the purified recombinant FPR is one such possibility. There are a few principal requirements for the crystallization of a protein [33]. First, there should be enough purified protein to produce a solution with a final concentration of milligrams per milliliters. In our report, we have demonstrated isolation of ~900 μg of purified FPR dissolved in 1 mL of chromatography buffer with no apparent solubility problems. Second, the preparation has to be homogeneous. Our recombinant FPR is translated from the DNA coding for the high affinity FPR, thereby overcoming the problem of co-presence of low-affinity FPR as found on neutrophils. Third, glycosylation has been shown to interfere with crystallization as well as introducing heterogeneity [39]; this obviously is not a problem when using our recombinant product. Finally, the stability of the protein in solution is also a factor in crystallization studies [37]; we did not observe any alteration of binding activity of the purified FPR throughout the whole project. Therefore, the purified rFPR described in this study may be an excellent candidate for crystallization studies.

An alternative biophysical approach is the use of 2D and 3D NMR spectroscopy of the purified FPR in solution. This is because the NMR spectrum of the bound FMLP to the receptor can be studied directly by employing isotope-edited NMR techniques [40–42]. Residues can be labeled with ^{13}C and ^{15}N using ^{13}C and ^{15}N glucose and NH_4NO_3, respectively. ^{15}N-H and ^{13}C-H correlation may then be used to identify the residues. Even if all the resonances of the complete FPR molecule (350 amino acids) may not be possible, residues that show changes in chemical shift due to ligand binding can be identified. We are currently evaluating the possibility of these various biophysical studies.

In conclusion, the preparation of the purified FPR will facilitate more extensive characterization of the receptor molecule and provide a model system to enhance our knowledge of the molecular structure and function of FPR, while the antibody will serve as a valuable tool in various assay procedures.

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