N-formylpeptides Induce Two Distinct Concentration Optima for Mouse Neutrophil Chemotaxis by Differential Interaction with Two N-formylpeptide Receptor (FPR) Subtypes: Molecular Characterization of FPR2, a Second Mouse Neutrophil FPR

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Summary

The N-formylpeptide receptor (FPR) is a G protein–coupled receptor that mediates mammalian phagocyte chemotactic responses to bacterial N-formylpeptides. Here we show that a mouse gene named Fpr-rs2 encodes a second N-formylpeptide receptor subtype selective for neutrophils which we have provisionally named FPR2. The prototype N-formylpeptide fMLF induced calcium flux and chemotaxis in human embryonic kidney (HEK) 293 cells stably transfected with FPR2. The EC50s, z5 mM for calcium flux and chemotaxis, were z100-fold greater than the corresponding values for mouse FPR-transfected HEK 293 cells. Consistent with this, fMLF induced two distinct concentration optima for chemotaxis of normal mouse neutrophils, but only the high concentration optimum for chemotaxis of neutrophils from FPR knockout mice. Based on these data, we hypothesize that high- and low-affinity N-formylpeptide receptors, FPR and FPR2, respectively, may function in vivo as a relay mediating neutrophil migration through the high and low concentration portions of N-formylpeptide gradients.

Key words: chemoattractant • inflammation • neutrophil • G protein–coupled receptor • phagocyte

The N-formylpeptides are cleavage products of bacterial and mitochondrial proteins, and serve as powerful chemoattractants for mammalian phagocytes (1–3). Based on their chemotactic actions, it has been hypothesized that N-formylpeptides attract phagocytes to sites of infection and therefore play an important role in antibacterial host defense (4). Like other chemoattractants, they act through seven-transmembrane-domain G protein–coupled receptors (5). Two functional N-formylpeptide receptors, designated FPR and FPR L1R, and one related putative receptor, designated FPR L2, have been identified by human gene cloning (6–11). FPR and FPR L1R bind the prototypical N-formylpeptide fMet-Leu-Phe (fMLF) with high and low affinity, respectively. The Kd for FPR is z3 nM, whereas that for FPR L1R is z100 times higher (10, 12). fMLF has been reported to induce calcium mobilization in cells transfected with FPR or FPR L1R and chemotaxis in cells transfected with FPR, but does not induce chemotaxis in cells transfected with FPR L1R when tested at concentrations as high as 2 mM (8, 10, 13, 14). FPR L1R has also been reported to bind lipoxin A4, a lipid derivative of arachidonate metabolism, with high affinity. Functions of lipoxin A4, mediated by FPR L1R include induction of GTPase activity and production of arachidonic acid; however, it has not been shown to induce calcium flux or chemotaxis (15). To our knowledge, FPR L1R is the only receptor whose agonists include both a peptide and a lipid. Recently, the range of potential biological actions of FPR L1R has become further complicated by the discovery that serum amyloid A (SAA), an acute phase reactant in inflammation, and T21, an ectodomain peptide of HIV-1 gp41, are also functional ligands for FPR L1R (16, 17). A second gp41 peptide named T20, which is a powerful blocker of HIV-1 entry currently in clinical trials, has been reported to be a functional ligand for FPR (18, 19).

To identify biological roles for these molecules, we have been studying their mouse counterparts. We previously characterized mouse FPR and have developed mice lacking this receptor by targeted gene deletion (20, 21). FPR−/− mice revealed an important role for FPR in innate host defense against Listeria monocytogenes (21). We have also
cloned five related mouse genes, designated Fpr-rs1, Fpr-rs2, Fpr-rs3, Fpr-rs4, and Fpr-rs5 (22). Extensive cross-hybridization analysis failed to find more than three related human genes, indicating that the FPR gene cluster has undergone differential lineage-specific expansion in mammals. This has created problems in defining orthologous relationships, particularly in defining the mouse FPR L1 orthologue. Fpr-rs1 and Fpr-rs2 are most similar to human FPR L1 (both show ~75% nucleotide identity in the open reading frame [ORF]), whereas the other three genes form a separate cluster with slightly lower sequence relatedness to FPR L1 (22). Like FPR L1, both Fpr-rs1 and Fpr-rs2 are expressed in leukocytes, although expression in specific leukocyte subsets has not previously been defined.

Chinese hamster ovary (CHO) cells transfected with Fpr-rs1 have been demonstrated to bind lipoxin A₄, and the encoded receptor has therefore been named LXAR (23). This suggested that Fpr-rs1 is the orthologue of FPR L1 but raised unanswered questions about its specificity for N-formylpeptides and about the ligand specificity of Fpr-rs2, as well as about the functional relationship of these molecules to FPR. Here we show that Fpr-rs2 encodes a second mouse neutrophil FMLF receptor subtype, which we now provisionally name FPR 2, which operates at higher concentrations of ligand than FPR.

**Materials and Methods**

**Mouse Leukocyte Purification.** Development of FPR knockout mice has been described previously (21). Mice used in this study were from FPR+/− × FPR+/− matings of an F1 backcross of FPR+/− 129/Sv with wild-type C57Bl/6 mice. Leukocytes were harvested from the peritoneal cavity of FPR+/− and FPR−/− mice after thioglycollate (TG) irritation, as described previously (21). Cells obtained after 3 h were >90% neutrophils, whereas cells obtained after 72 h were >90% macrophages, as determined by the morphologic appearance of Diff-Quick-stained preparations.

**RNA Analysis.** Cells were lysed, and total RNA was prepared using the RNA STAT-60 protocol (Tel-Test, Inc.). RNA (10 µg) was separated on a denaturing agarose gel, and Northern blots were prepared by standard methods (24). Blots were hybridized with a full-length Fpr-rs2 ORF probe, which was labeled with ³²P-dCTP using a random-primer DNA labeling kit (Boehringer Mannheim). To control for RNA loading, the blots were also probed separately with a 49-bp oligonucleotide from the 5′ terminal end of the actin gene, which was labeled with a DNA 5′ end labeling kit (Boehringer Mannheim).

Creation of a Cell Line Expressing FPR 2. The Fpr-rs2 ORF was amplified from genomic clone 7 (22) using the upper strand primer 5′-ATATAAGCTTGGCCACCATGATTTATAGAA-TGATTGATGAAAGAATCTTCACCTGCACTTCTGCCATGAAATG-TTC-3′, which contains a Flag epitope tag (underlined), an EcoRI site (italicized), and an ATG initiating codon (bold); and lower strand primer 5′-CGCTGAGTCATCCGAGG-GGCCTTAACTCATGCTG-3′, which contains an Xhol site (italicized sequence) and the termination codon (bold). The 1.1-kb PCR fragment was then ligated into pCR2.1. Sequence fidelity was verified, and the correct insert was then subcloned between the NotI and XhoI sites of pcDNA3 (Invitrogen). Human embryonic kidney (HEK) 293 cells were maintained in DMEM with 10% FBS. 10⁶ cells in log phase were electroporated with 20 µg of plasmid DNA using a GenePulser (Bio-Rad Laboratories). Cell colonies resistant to 2 µg/liter G-418 (GIBCO BRL) were isolated and expanded in DMEM with 10% FBS and 2 ng/liter G-418.

Intraepithelial [Ca²⁺] M measurements. Cells (~10⁷/ml) were incubated in HBSS and 2.5 µM Fura-2 AM (Molecular Probes, Inc.) for 30–60 min at 37°C in the dark. The cells were then washed with HBSS and resuspended at 10⁷ cells/ml. 4 × 10⁶ cells were stimulated in a total volume of 2 ml in a continuously stirred cuvette at 37°C in a fluorimeter (Photon Technology, Inc.). FMLF, MLF, and recombinant C5a were obtained from Sigma Chemical Co. The chemokines IL-8, macrophage-inflammatory protein (MIP)-1β, and monocyte-chemotactic protein (MCP)-3 were obtained from Peprotech. The data were recorded every 200 ms as the relative ratio of fluorescence emitted at 510 nm after sequential excitation at 340 and 380 nm. For some experiments, cells were incubated with 250 ng/ml pertussis toxin for 4 h before functional assay.

**Chemotaxis.** HEK 293 cells were detached from flasks by replacing media with 0.05% trypsin (Quality Biological) and incubating at room temperature for ~1 min. DMEM containing 20% FBS was added, and the cells were harvested, pelleted, and resuspended at 10⁵ cells/ml in RPMI 1640 supplemented with 1% BSA and 20 mM Hepes. FMLF was loaded at varying concentrations in the lower compartment of a 48-well microchemotaxis chamber (NeuroProbe). To distinguish chemotaxis from chemokinesis, additional experiments were carried out in which an equal concentration of FMLF was tested simultaneously in the upper and lower compartments. The chamber was soaked in 1% SDS overnight and washed before each experiment. A polyclonal antifactor IX antibody (10- and 3-μm pores for HEK 293 cells and neutrophils, respectively) was used. For experiments with HEK 293 cells, the filters were coated with 0.05 mg/ml rat tail collagen in RPMI 1640 and 70 mM Hepes for 2 h and dried before each experiment. The filter, coated side down, was placed between the upper and lower compartments of the chamber, and 50 µl of 10⁵ HEK 293 cells/ml was loaded in the upper compartment. The chemotaxis chamber was incubated at 37°C, 100% humidity, and 5% CO₂ for 5 h for HEK 293 cells and 45 min for neutrophils. The filter was then removed, washed, dried, and stained. Cells that migrated through the filter were counted microscopically under high power. All conditions were tested in triplicate.

**Results**

Fpr-rs2 Is Expressed in Mouse Phagocytes. Previously, we reported that Fpr-rs2 is expressed in unfractionated peripheral blood leukocytes (22). To determine the expression pattern in finer detail, we used Northern blot hybridization to probe total RNA from peritoneal cells elicited 3 and 72 h after instillation of TG. The elicited peritoneal cell populations were markedly enriched in neutrophils and macrophages at 3 and 72 h, respectively (~90% pure). The residual 10% of cells were mainly mononuclear cells and neutrophils in the 3- and 72-h cell populations, respectively. Specific mRNA bands were detected in both the 3- and 72-h TG-elicited peritoneal cells. In both cases, two classes of transcripts
(~1.5 and 1.9 kb) were observed, and the signal strength was similar for each class within each sample. However, signals from the 3-h cells were much stronger than from the 72-h cells (Fig. 1). Note that 72-h RNA was deliberately overloaded relative to 3-h RNA, as revealed by hybridization with an actin probe. A reasonable interpretation of these results, consistent with the functional data that follows, is that Fpr-rs2 is primarily expressed in neutrophils, and the weak signal in the 72-h cells is due to neutrophils, which make up a small minority of the total cell population. An alternative explanation is that monocytes express Fpr-rs2 in the circulation, but not after extravasation.

Fpr-rs2 Encodes a Low-affinity G Protein–coupled FPR. To assess whether Fpr-rs2 encodes a functional FPR, we performed a gain-of-function genetic test by measuring fMLF-induced intracellular Ca$^{2+}$ flux in HEK 293 cell lines stably transfected with expression plasmids containing the Fpr-rs1 or Fpr-rs2 ORFs.

In the initial screen, we tested 16 G-418–resistant HEK 293 cell colonies derived from the same transfection, all of which responded to 10 μM fMLF. In contrast, nonformylated MLF at 1 μM, C5a at 10 nM, and the chemokines IL-8, MIP-1β, and MCP-3 at 100 nM or greater did not induce a response. Also, Fpr-rs1–transfected cells, maintained in the same selective conditions, consistently failed to respond to fMLF at concentrations as high as 100 μM (Fig. 2 A). Higher concentrations could not be tested for technical reasons due to the hydrophobicity of fMLF. Based on this result, we infer that Fpr-rs2 encodes an FPR, which we provisionally name FPR2.

Four cell lines that gave particularly strong responses, designated L4-7, L4-14, L4-15, and L4-16, were selected for further study. In each case, the calcium flux responses were concentration dependent and saturable, with an EC50 of 5.3 ± 0.3 μM (n = 5, each cell line tested at least once; Fig. 2 C). HEK 293 cells transfected with mouse FPR also exhibited a calcium flux response to fMLF that was concentration dependent and saturable; however, the EC50, ~50 nM, was much lower than for FPR2 (Fig. 2 C). This value is consistent with that previously reported for expression of mouse FPR in frog oocytes in a calcium-release assay (20).

When FPR2-transfected cells were sequentially stimu-
lated with 100 μM fMLF, no response was observed after the second stimulation, suggesting complete receptor desensitization by the first stimulus (Fig. 2 A). The fMLF response could also be abolished by pretreatment of FPR 2-expressing cells with pertussis toxin, suggesting that the receptor is coupled to a G₁-type G protein (Fig. 2 B). As a control of cell integrity, we stimulated toxin-treated cells with 10 μM ATP, which induced a calcium flux at levels equivalent to the untreated cells.

To test the mechanism of fMLF induction of calcium flux in FPR 2-transfected cells, we carried out radioligand binding assays using a ³H-fMLF probe. Although human and mouse FPR-expressing cells exhibited specific binding, cells expressing FPR 2 did not (data not shown). This is not surprising, since the highest concentration of ³H-fMLF that could be meaningfully tested was 640 nM, which is below the threshold of detection of calcium flux induced by fMLF in FPR 2-transfected cells. In contrast, the threshold for induction of calcium flux by fMLF in human and mouse FPR-transfected cells is ~0.5 and 10 nM, respectively. We conclude that FPR 2 is most likely a low-affinity FPR.

FPR 2 is a chemotactic receptor. Given the reactivity of FPR 2 to fMLF observed in the calcium flux assay, we next tested its ability to mediate chemotaxis. FPR 2-expressing cells migrated in a concentration-dependent manner in response to fMLF with a threshold of ~1 μM. The EC₅₀ was ~5 μM. In all experiments, the upward phase of the concentration-response curve was consistently superimposable with that of the calcium flux assay for the same FPR 2-expressing cell line. Mouse FPR-expressing HEK 293 cell migration followed a clear-cut bell-shaped concentration-response curve whose EC₅₀ was shifted ~10–100-fold to the left relative to the FPR 2 curve (Fig. 3). The activity was specific for both receptors, since HEK 293 cells transfected with the related gene Fpr-rs1 and selected with G-418 did not exhibit concentration-dependent migration in response to fMLF when tested with concentrations ranging from 0.1 nM to 100 μM (Fig. 3 B). To distinguish chemotaxis from chemokinesis, equal concentrations of fMLF were added to the upper and lower chambers of the chemotaxis apparatus. In this configuration, no dose-dependent cell migration was observed (Fig. 3 A). Therefore, FPR 2 can not only cause intracellular signaling, but can also use those signals to elicit a chemotactic action by the cell.

FPR 2 May Mediate Mouse Neutrophil Calcium Flux and Chemotaxis. To determine whether FPR 2 may operate as a second mouse neutrophil fMLF receptor in primary cells, we examined fMLF responses by cells from FPR knockout mice. Previously, we reported that neutrophils from these mice failed to respond to fMLF either in calcium flux or chemotaxis assays at concentrations as high as 1 μM, and noted that this correlated well with the concentration-response relationship for HEK 293 cells expressing mouse FPR in the calcium flux assay (21).

However, having now discovered that the threshold for calcium flux in FPR 2-expressing cells was ~1 μM, we re-tested neutrophils from these animals at higher concentrations and observed a concentration-response relationship that was virtually identical for FPR⁻/⁻ neutrophils versus HEK 293 cells expressing FPR 2 for both calcium flux and chemotaxis (Figs. 2–4). The EC₅₀ value in FPR⁻/⁻ neutrophils was ~6 μM for calcium flux, in close agreement with the value of 5.3 μM cited above for FPR 2-expressing HEK 293 cells (compare Figs. 2 C and 4 A). The chemotaxis concentration-response curves were also very similar (compare Figs. 3 B and 4 B).

Calcium flux and chemotaxis experiments were also performed on neutrophils from wild-type mice using an expanded concentration range relative to what was used in our previous report, 1 nM to 100 μM (Fig. 4). In the calcium flux assay, the response saturated at ~1 μM fMLF, which corresponds to the saturation concentration for

**Figure 3.** FPR 2 is a chemotactic receptor. HEK 293 cells stably transfected with mouse FPR, FPR 2, or Fpr-rs1 were incubated in a microchemotaxis chamber for 5 h, and the number of migrating cells was counted. Data shown are from a single experiment representative of more than five separate experiments in each panel with a consistent pattern. (A) FPR 2 transfecants with equal concentration of fMLF in the upper and lower chambers (fMLF/fMLF, open circles), or with fMLF in only the lower chamber (Medium/fMLF, filled circles). *Statistically significant difference between the points shown and baseline migration in the absence of fMLF, P < 0.0005 by Student’s t test. (B) FPR transfecants (circles) and Fpr-rs1 transfecants (squares).
Discussion

In this study, we have identified dual concentration-response optima for normal mouse neutrophils in chemotaxis induced by the proinflammatory chemoattractant fMLF. For leukocytes, chemoattractant concentration–response relationships are classically described by a bell-shaped curve with a single optimum; the presence of a second optimum as we have described is highly unusual. The functional characteristics of FPR versus FPR2, the second mouse neutrophil low-affinity fMLF receptor subtype that we have identified, in conjunction with analysis of neutrophils from mice lacking the high-affinity fMLF receptor FPR, strongly suggest a molecular explanation for this anomaly in which FPR and FPR2 account for the low and high concentration–response optima, respectively.

The significance of this result relates to the dilemma of how leukocytes navigate through the highest portions of chemotactant concentration gradients in which their high-affinity chemoattractant receptors are likely to become deactivated through receptor phosphorylation and/or sequestration mechanisms (25, 26). Our data suggest the hypothesis that distinct high- and low-affinity receptors for the same chemoattractant may work as a relay to sensitize the cell throughout the gradient, allowing it to arrive at the focus of inflammation.

Most work on leukocyte chemotactic receptors has focused on the identification of receptors that bind ligand in the low nanomolar range, so-called “high-affinity receptors” because binding is easy to measure with available radioligand probes. However, potentially important low-affinity ligand–receptor interactions have also been identified, such as multiple CXC chemokines for CXCR1 (27) and MIP-1β and MCP-1 for CCR1 (28). In this regard, the CXC chemokine neutrophil-activating peptide 2 (NAP-2) is particularly interesting since, like fMLF in our study, it appears to activate neutrophil chemotaxis via two concentration-response optima by high-affinity binding to CXCR2 and low-affinity binding to CXCR1 (29).

Alternative mechanisms of navigation may also exist, such as usage of high-affinity receptors at different points during the migration path through differential spatial expression of cognate chemoattractants, or by receptor recycling, or by a combination of these mechanisms. In this regard, chemokines may be particularly important for fine-tuning leukocyte migration to inflammatory sites.

In humans, the two functional FPRs, FPR and FPR1R, also have high and low affinity, respectively, for fMLF (7, 10, 12). Consistent with this, in calcium flux assays, fMLF is ~100-fold more potent at FPR versus FPR1R in vitro. This hierarchy is analogous to that for mouse FPR and FPR2. However, one significant difference is that, to date, FPR1R has not been reported to be a chemotactic receptor, and its biological function remains unknown. Therefore, the discovery of FPR2 represents the first time that two chemotactic FPRs have been observed in a single species.

FPR1R also binds lipoxin A4 with high affinity (15), to our knowledge the only example of a receptor with both peptide and lipid ligands. In mice, the related gene Fpr-rs1 encodes a receptor, LXA4R, that binds lipoxin A4 (23). Therefore, it would
appear that differential expansion of an ancestral FPR gene occurred during evolution between humans and mice, resulting in two mouse receptors that split the functions of human FPR1R: FPR2 mediates responses to N-formylpeptides, whereas LXA4R mediates responses to lipoxin A4. This theory will need to be tested by additional experiments addressing the specificity of FPR2 for lipoxin A4 and LXA4R for fMLF. In addition, these receptors will need to be tested for their specificity for SAA and HIV-1 T21, which act at human FPR1R, and HIV-1 T20, which acts at FPR (16–18). Moreover, alternative N-formylpeptides besides fMLF must be considered as potential physiological ligands for both FPR and FPR2.

In conclusion, since FPR and FPR2 are both expressed in neutrophils and are differentially sensitive to fMLF, we propose that they may act sequentially during the inflammatory response: FPR recruits neutrophils in the low concentration portion of an N-formylpeptide gradient, whereas FPR2 operates closer to the inflammatory focus where concentrations are expected to be highest and FPR is more likely to be desensitized. We are currently developing an FPR2 knockout mouse to test this hypothesis further, as well as to test the physiological role of FPR2 and its relationship to human chemoattractant signaling.

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