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F2L, a Peptide Derived from Heme-Binding Protein, Chemoattracts Mouse Neutrophils by Specifically Activating Fpr2, the Low-Affinity N-Formylpeptide Receptor

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F2L (formylpeptide receptor (FPR)-like (FPRL)-2 ligand), a highly conserved acetylated peptide derived from the amino-terminal cleavage of heme-binding protein, is a potent chemoattractant for human monocytes and dendritic cells, and inhibits LPS-induced human dendritic cell maturation. We recently reported that F2L is able to activate the human receptors FPRL-1 and FPRL2, two members of the FPR family, with highest selectivity and affinity for FPRL2. To facilitate delineation of mechanisms of F2L action, we now have attempted to define its mouse receptors. This is complicated by the nonequivalence of the human and mouse FPR gene families (three vs at least eight members, respectively). When cell lines were transfected with plasmids encoding the eight mouse receptors, only the one expressing the receptor Fpr2 responded to F2L (EC50 ~400 nM for both human and mouse F2L in both calcium flux and cAMP inhibition assays). This value is similar to F2L potency at human FPRL1. Consistent with this, mouse neutrophils, which like macrophages and dendritic cells express Fpr2, responded to human and mouse F2L in both calcium flux and chemotaxis assays with EC50 values similar to those found for Fpr2-expressing cell lines (~500 nM). Moreover, neutrophils from mice genetically deficient in Fpr2 failed to respond to F2L. Thus, Fpr2 is a mouse receptor for F2L, and can be targeted for the study of F2L action in mouse models.

Transfectants were also generated in HEK 293T and HEK 293 cells as controls. Dose-response curves were obtained using PcaeqG15 cell line. Receptor open reading frames were amplified by PCR from mouse genomic DNA for Fpr1, Fpr-rs1 (two variants named 1a and 1b), Fpr-rs2, Fpr-rs3, Fpr-rs4, Fpr-rs6, and Fpr-rs7 (24, 25), inserted into the eu-karyotic expression vectors pcdNA3 (Invitrogen Life Technologies) and pEFIN3 (Euroscreen), and verified by direct DNA sequencing. The empty vectors and the human FPR2 constructs (1) were used as controls. CHO-K1 cells expressing G\_alpha16 and apaoepoerin (PcAeqG15; Euroscreen) were transfected with the pEFIN3 constructs using FuGene 6. Transfectants were also generated in HEK 293T and HEK 293 cells as described previously (29, 35).

**Materials and Methods**

In vitro expression of the mouse family of FPR-like receptors

Receptor open reading frames were amplified by PCR from mouse genomic DNA for Fpr1, Fpr-rs1 (two variants named 1a and 1b), Fpr-rs2, Fpr-rs3, Fpr-rs4, Fpr-rs6, and Fpr-rs7 (24, 25), inserted into the eu-karyotic expression vectors pcdNA3 (Invitrogen Life Technologies) and pEFIN3 (Euroscreen), and verified by direct DNA sequencing. The empty vectors and the human FPR2 constructs (1) were used as controls. CHO-K1 cells expressing G\_alpha16 and apaoepoerin (PcAeqG15; Euroscreen) were transfected with the pEFIN3 constructs using FuGene 6. Transfectants were also generated in HEK 293T and HEK 293 cells as described previously (29, 35).

**Mice**

Fpr-deficient mice have been described previously (27). These mice have been backcrossed for 10 generations to the C57BL/6 strain from Taconic Farms, which was also used as the control strain. Detailed description of the generation and phenotype of Fpr2-deficient mice will be reported separately (J. M. Wang, manuscript in preparation). Wild-type (wt) littermates from F1 matings of heterozygotes on a mixed C57BL/6 and Sv/129 background were used as controls for Fpr2-deficient mice. All procedures were reviewed and approved by the Animal Care and Use Committee at National Institute of Allergy and Infectious Diseases, National Institutes of Health, or by the Commission d’Ethique du Bien-Etre Animal of Université Libre de Bruxelles (ULB).

**Preparation of mouse neutrophils**

Neutrophils were obtained from the peritoneal cavity of 6-wk-old wt C57BL/6 mice 3 h after i.p. injection of 2 ml of 3% thioglycolate solution. The cell population was consistently composed of >90% neutrophils, as determined by flow cytometry analysis (CD11b/Gr-1-positive population). In the experiments using bone marrow-derived LPS-treated neutrophils, cells were obtained by flushing tibias and femurs of Fpr-deficient, Fpr2-deficient, and wt mice with PBS. The isolated bone marrow was suspended in DMEM supplemented with 10% FBS, 20 mM HEPES, and 500 ng/ml LPS (Sigma-Aldrich), and cultured overnight at 37°C. The cells were washed with PBS and the remaining RBC were lysed with ACK lysis buffer. The cell population was consistently composed of >85% neutrophils.

**Aequorin assay**

A calcium release assay based on the luminescence of mitochondrial aequorin in CHO-K1 cells expressing G\_alpha16 and apaoepoerin (PcAeqG15) transfected with pEFIN3 constructs was performed as described previously (36). For screening, G418-resistant cells (mixed clonal populations) were transfected with various concentrations of human F2L (2 nM, 20 nM, 200 nM, and 2 \mu M; synthesized by the Laboratory of Chemistry at ULB, Brussels, Belgium (11)). A total of 2 \mu M W hexapeptide (WKYMVM: ULB), 1 \mu M IMLF (Neosystem), and 1 \mu M humanin (Peptides International) were used as controls. Dose-response curves were obtained using PcAeqG15 cell line. For cAMP determinations, the pcdNA3 constructs were transfected into HEK 293T cells using the calcium phosphate precipitation method (35). Forty-eight hours after transfection, culture medium was removed and replaced by Krebs-Ringer-HEPES buffer (KRH) for 30 min. Thereafter, cells were incubated for 60 min in fresh KRH supplemented with 25 \mu M phosphodiesterase inhibitor Rolipram (Laboratoire Logeais) and 10 \mu M forskolin (FSK) with or without 2 \mu M F2L. The medium was discarded and replaced with 0.1 M HCl, and the cell extracts were dried under vacuum, resuspended in water, and diluted appropriately for cAMP measurements according to Broeker et al. (37). Duplicate samples were assayed in all experiments, and the results are expressed in picomoles per milliliter. Dose-response curves were performed using a homogeneous time-resolved fluorescence kit (Cis Bio International). In brief, cells (stable clones of CHO-K1 cells coexpressing Fpr2, G\_alpha16 and apaoepoerin) were detached, resuspended in KRH containing 1 \mu M 3-isobutyl-1-methylxanthine, and stimulated with 5 \mu M FSK alone or together with various concentrations of agonists for 30 min at 37°C. The reaction was stopped by the successive addition of cAMP-XL665 and anti-cAMP cryptate diluted in lysis buffer. The plates were incubated for 60 min at room temperature and read on the Rubystar fluorometer (Labtech). A calibration curve was obtained by plotting δ F\% vs CAMP concentrations. Dose-response curves were fitted using the Prism version 4 program (GraphPad).
Intacellular calcium measurements

Thioglycolate-elicited neutrophils were loaded by incubation with 5 μM fura-2 (Molecular Probes) at 37°C for 30 min in the dark. Subsequently, the cells were washed and resuspended at 5 × 10⁶/ml in HBSS with 0.1% BSA. A total of 2 ml of loaded neutrophils was transferred into a quartz cuvette, which was placed in a luminescence spectrometer (LS50B, PerkinElmer). Calcium flux in response to agonists was measured by recording the ratio of fluorescence emitted at 510 nm after sequential excitation at 340 and 380 nm. Intracellular calcium measurement for HEK 293 cells and bone marrow neutrophils was performed in a Benchtop Scanning Fluorometer and Integrated Fluid Transfer Workstation (FlexStation; Molecular Devices). A total of 2.5 × 10⁵ cells in 100 μl of HBSS with 20 mM HEPES buffer was loaded into a 96-well measuring plate together with 100 μl of fluorescent dye (FLIPPER calcium 3 assay kit component A; Molecular Devices), incubated for 30 min at 37°C, and then centrifuged for 5 min. Intracellular calcium concentration changes in response to added agonists were recorded as relative fluorescence units.

Chemotaxis

Chemotaxis assays of bone marrow neutrophils were performed in a 48-well microchemotaxis chamber (NeuroProbe). Neutrophils were suspended at 10⁶ cells/ml in RPMI 1640 supplemented with 1% BSA and 20 mM HEPES. Agonists were loaded at varying concentrations in a final volume of 28 μl in the lower compartment of the chamber. A polycarbonate filter (3-μm pore size) was placed between the upper and lower compartments of the chamber, and 50 μl of 10⁶ cells/ml were loaded in the upper compartment. The chemotaxis chamber was incubated at 37°C, 100% humidity, and 5% CO₂ for 45 min. The filter was then removed, washed, fixed, and stained with HEMA 3 Stain Set (Fisher Diagnostics). Cells that migrated to the bottom side of the filter were counted microscopically. All conditions were tested in triplicate. The results are presented as chemotaxis indices, representing the fold increase in the number of migrating cells in response to stimuli over the spontaneous cell migration (in response to control medium).

Results

F2L specifically activates Fpr2

To identify mouse F2L receptor(s), we screened receptor-transfected cell lines using two functional responses: 1) inhibition of FSK-induced cAMP accumulation and 2) intracellular calcium flux. Cells expressing human FPRL2 or mouse Fpr2 demonstrated a modest but significant inhibition of FSK-induced cAMP accumulation when stimulated with 2 μM F2L (Fig. 1A), whereas cells transfected with plasmids encoding the other receptors did not respond. In the aequorin-based calcium flux assay, mixed clonal populations of CHO-K1 cells transfected with plasmids encoding the eight mouse receptors selected with G418 were used in the initial screening. In these experiments, fMLF, W hexapeptide, and humanin were used as control agonists, and pcAeqG15 cells transfected with pcDNA3 (mixed population) or transfected with a FPRL2 construct (clonal population) were used as negative and positive control cell lines, respectively. We observed specific activation of cells expressing Fpr2 after stimulation with 2 μM F2L, whereas cells transfected with plasmids encoding the other FPR family members did not respond. As expected, the cell lines expressing human FPRL2, or mouse Fpr and Fpr2 responded appropriately to their known agonists: FPRL2 to F2L and humanin, Fpr

ATP (A) or 10 μM FSK (B). Measurements are performed in triplicate for cAMP assay, and duplicate for aequorin assay, and data are presented as mean ± SEM. Data are from a single experiment representative of at least three independent experiments. C and D, Flexstation-based calcium flux assay. A clonal population of HEK 293 cells expressing Fpr (C) or Fpr2 (D) was analyzed using agonists and concentrations indicated in the inset of each panel. Data are from a single experiment representative of at least three independent experiments. E, Pharmacological parameters for agonists of mouse Fpr2 and human FPRL2. The indicated values were derived from data obtained using the aequorin assay on clonal populations of Fpr2- and FPRL2-expressing CHO-K1 cells. pEC₅₀ = [−log (EC₅₀)].
to fMLF and W hexapeptide, and Fpr2 to fMLF, W hexapeptide, and humanin. The other cell lines could not be validated functionally due to the absence of known agonists (Fig. 1B). Results were also obtained independently using the Flexstation system for intracellular calcium flux measurement in HEK 293 cells stably transfected with plasmids for Fpr, Fpr2, Fpr-rs1, and Fpr-rs6 (Fig. 2, C and D, and data not shown). Fpr2-expressing cells responded to F2L in a concentration-dependent manner, whereas Fpr-expressing cells, which responded to fMLF, failed to respond to F2L at any concentration tested.

Pharmacological analysis of the Fpr2-F2L interaction

To study the interaction between Fpr2 and F2L in greater detail, we selected clones of CHO-K1 cells stably expressing Fpr2 from parent cell lines expressing apoaequorin and Ga16. Dose-response curves were established both in the aequorin-based calcium flux assay and in a cAMP accumulation assay, and the resulting functional parameters were derived (Fig. 2, A, B, and E). We observed that F2L appears as a relatively low potency agonist for Fpr2, with an EC50 of 400 ± 30 nM in the aequorin-based assay, which is substantially higher than the corresponding value for FPRL2 (10 nM) (1). Similar results were obtained in the cAMP accumulation assay, with an EC50 of 187 ± 44 nM for F2L activity at Fpr2 and 5 nM at FPRL2. A similar EC50 for intracellular calcium flux, ~500 nM, was obtained when F2L was used to stimulate Fpr2-expressing HEK 293 cells (Fig. 2, C and D).

The low potency of human F2L at mouse Fpr2 relative to its activity at human FPRL2 could be due to species specificity of the ligand. To address this directly, we tested the corresponding peptide derived from the mouse HBP sequence, which differs from human F2L by only 1 aa, a lysine to arginine substitution at position 6. Mouse F2L was found to activate Fpr2 in the aequorin assay with an EC50 of 665 ± 190 nM, which did not differ significantly from the value for human F2L (Fig. 2E). Likewise, in the aequorin assay mouse and human F2L were equipotent at human FPRL2. As a positive control, humanin was able to activate Fpr2 (EC50: 700 ± 240 nM) and FPRL2 (Fig. 2E).

F2L activates mouse neutrophils

To test whether native Fpr2 also functions as an F2L receptor, we focused on primary mouse neutrophils, which are known to express Fpr2 (29). Neutrophils recruited to the peritoneal cavity by thioglycollate injection were recovered by lavage and used to measure intracellular Ca2+ flux in response to human F2L (Fig. 3A). As controls, we used different agonists known to activate receptors expressed on neutrophils, such as IL-8 (data not shown), ATP, and fMLF. The cells responded to human F2L in a concentration-dependent manner with an EC50 ~500 nM (Fig. 3A), which agrees with the results obtained using Fpr2-expressing cell lines. Of note, high concentrations of F2L were able to desensitize the response of the cells to 2 μM fMLF but not to ATP, suggesting that the two agonists either share receptors or use unique receptors that can be cross-desensitized, or both. The third possibility is supported by our evidence that fMLF activates both Fpr and Fpr2 (29), whereas F2L activates only Fpr2. These results were confirmed and extended using bone marrow-derived neutrophils with or without pretreatment of the cells with LPS and both mouse and human F2L (Fig. 3, B and C, and data not shown). LPS is known to up-regulate membrane expression of Fpr2 (30), and greatly increased cell responsiveness to both mouse and human F2L.

FIGURE 3. F2L induces calcium flux in mouse neutrophils. A, Thioglycollate-elicited peritoneal neutrophils. Cells were loaded with fura-2, and fluorescence changes were measured in a stirred cuvette stimulated at the times indicated by arrows with the indicated agonists and concentrations. Data are from one experiment representative of two independent experiments.

B and C, Bone marrow-derived neutrophils. Cells were treated with LPS (500 ng/ml) in vitro overnight, which has been reported to increase expression of Fpr2, then stimulated with human (B) or mouse (C) F2L at the concentrations indicated in the inset. Calcium flux was monitored by fluorescence changes using the Flexstation system. Data are from one experiment representative of at least two independent experiments.

F2L activation of mouse neutrophils is specifically mediated by Fpr2: calcium flux analysis

To address the biological relevance of F2L action at Fpr2, we first performed calcium flux assays using neutrophils derived from wt,
Fpr-deficient, and Fpr2-deficient mice. Fpr-deficient neutrophils displayed the expected shift in sensitivity to fMLF, being only able to respond to high concentrations of the peptide, presumably through the activation of Fpr2 (Fig. 4). However, they responded to F2L with a dose dependence identical with that of wt cells (Fig. 4). In contrast, Fpr2-deficient neutrophils were totally unable to respond to human and mouse F2L. The loss of function was highly specific because the cells responded normally to fMLF, acting through Fpr, and the chemokine MIP-2, acting through the receptor CXCR2 (Fig. 4 and data not shown). To address whether the deletion of the Fpr2 gene affected the expression levels of other Fpr-related genes, we performed RT-PCR for Fpr, Fpr-rs1, Fpr2, and Fpr-rs3–7 on bone marrow-derived neutrophils pretreated with LPS. Cells derived from wt mice expressed Fpr and Fpr2 mRNA, whereas cells derived from Fpr2-deficient animals expressed only Fpr mRNA, as expected. We did not observe a difference in Fpr expression between wt and Fpr2-deficient neutrophils. In our experimental conditions, we did not observe Fpr-rs1, and Fpr-rs3–7 mRNA expression (data not shown).

F2L chemoattracts mouse neutrophils by activating Fpr2

Chemotaxis experiments were performed using thioglycollate-elicited (data not shown) and LPS-treated bone marrow neutrophils from wt and Fpr2-deficient mice with similar results. Human F2L was able to chemoattract wt neutrophils in a dose-dependent manner, with a threshold concentration of 500 nM. In contrast, neutrophils from Fpr2-deficient mice were totally unresponsive (Fig. 5). The loss of function was specific because Fpr2-deficient neutrophils were able to migrate to fMLF, as expected, due to expression of Fpr.

Discussion

In this study, we have demonstrated that the G protein-coupled receptor and FPR family member Fpr2 is a specific mouse receptor for F2L, an N-acetylated peptide corresponding to the N-terminal 21 aas of HBP. We have also demonstrated that F2L is a mouse neutrophil chemoattractant and that this activity is mediated specifically by Fpr2. These data extend our previously published identification of monocytes and dendritic cells as human cells that are chemotactically responsive to F2L, and of FPRL1 and FPRL2 as human FPR family members with low and high selectivity and affinity for F2L, respectively. Moreover, they provide a means to study the mechanism of action of F2L and Fpr2 in vivo.

F2L potency at Fpr2 is similar to its potency at human FPRL1. This is not surprising because Fpr2 is a structural homolog of FPRL1 and shares many agonists with it. However, a major question that our study has not answered is whether mouse has a second receptor with higher selectivity for F2L, that is, a functional orthologue of human FPRL2. To date, no such receptor has been identified. This may have to do with the unbalanced evolution of the FPR gene cluster in different species. Examination of the FPR receptor dendrogram (24, 25) shows three evolutionarily distinct clusters. The first contains human FPR and mouse Fpr, which clearly form an orthologous pair. A second contains five mouse genes, Fpr-rs3–7, all of which encode orphan receptors, but not any human genes. The third contains human FPRL1 and FPRL2, mouse Fpr2, and mouse ALX (the lipoxin A4 receptor and the product of the gene Fpr-rs1). Close inspection of this third cluster shows that mouse ALX and Fpr2 are on a structural level significantly more closely related to human FPRL1 than to human FPRL2. Furthermore, on a functional level, they appear to split the agonists of FPRL1: LXA4 in the case of ALX, and fMLF and multiple other peptide and protein agonists as well as LXA4 in the case of Fpr2. Thus, absence of a second mouse receptor with higher selectivity for F2L may not be surprising. It is important to...
note that our functional screen of FPR family members in transfected cells was not able to independently verify the surface expression of Fpr-rs1 and the five orphan members of the gene subfamily, so that it is still possible that they scored as false negatives in the F2L functional screen. Nevertheless, if one of these receptors, or any other receptor outside this family, is a second F2L receptor, our ex vivo analysis suggests strongly that it does not operate in our preparation of LPS-treated bone marrow-derived mouse cells, (~85% neutrophils). Additional studies will be needed to determine whether Fpr2 plays an exclusive role in mediating F2L action in other mouse cell types, as it does in neutrophils.

Mouse and human FPR have a similar distribution but differ by more than two logs in affinity for iMLF. Fpr2 (mouse) and FPR1 (human), while having similar selectivity for F2L, are both expressed by neutrophils, but Fpr2, not FPR1, is also expressed on dendritic cells. FPR2 (human) is expressed on dendritic cells, but not on neutrophils, and is two logs more sensitive than Fpr2 to F2L, while not responding to iMLF at all. These differential properties could not be predicted from simple inspection of the amino acid sequence relationships among these receptors.

The amino acid sequence identity of Fpr2 with FPR1 is 76%, but only 63% with FPR2. Several regions that might be involved in receptor/ligand interactions, in particular the second extracellular loop, are well conserved between FPR2 and Fpr2, while differing from ALX.

The biological functions of FPR receptors are not understood precisely yet. The antimicrobial role of FPR has been clearly demonstrated thanks to animal models. FPR might also be involved in the immune response to cell death, as mitochondrial formylated peptides are released in these circumstances as well. In contrast, when stimulated with annexin 1, the receptor was reported to be involved in an anti-inflammatory response (38). The role of FPR1 is even more difficult to define, due to the large number of ligands. Several antagonist, although not necessarily mutually exclusive, roles have been attributed to this receptor. It is probably involved in proinflammatory responses, such as those elicited by microorganisms releasing activating peptides, or by antimicrobial cathelicidins, among other factors. However, through the binding of LXA4 and annexin 1, FPR1 has also been implicated in anti-inflammatory responses. It might also play an important role in amyloidogenic diseases, such as Alzheimer’s disease, prion diseases, and chronic inflammation, as it responds to AP42, PrP106-126, and serum amyloid A, but it can also be triggered by the neuroprotective peptide humanin. Once again, it is not clear at this stage whether FPR1 acts as a protective or enhancing factor in these diseases. The description of Fpr2 as a mouse receptor responding to these agonists, and expressed, among other sites, in brain microglia, as well as the generation of a Fpr2 knockout (ko) mouse model for this receptor, will enable research of these clinically relevant issues.

As far as FPR2 is concerned, its biological function is essentially unknown, partly because no specific naturally occurring agonist was available until recently. The description of F2L and human as strong agonists may help to resolve this issue. However, an important challenge will be to understand the circumstances in which the F2L peptide is generated and released. HBP is a ubiquitously expressed intracellular protein that lacks a signal peptide. Its cleavage product, F2L, is a potent chemoattractant for macrophages and dendritic cells. It is thus an interesting candidate for mediating attraction of APCs toward necrotic and apoptotic cells, as has been shown for annexin 1 and high mobility group box-1 protein 1. F2L might be generated inside or outside cells, and either secreted by nonconventional mechanisms, or released passively as a result of cell death. The identification of the protease(s) involved, and of the secretion mechanism, would provide very useful hints regarding the role of the FPR2/F2L couple, and this is currently under investigation.

Although Fpr2 cannot be considered as a strict orthologue of FPR2, the identification of Fpr2 as the only detectable mouse receptor for F2L provides an important tool for designing studies of its in vivo functions. The relevance of such studies is given added weight by the fact that HBP is highly conserved among species and mouse and human F2L are equivalent in vitro.

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Disclosures
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