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DIFFERENTIAL EXPRESSION OF MEMBERS OF THE *N*-FORMYLPEPTIDE RECEPTOR GENE CLUSTER IN HUMAN PHAGOCYTES

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The human genes for two *N*-formylpeptide phagocyte chemoattractant receptors (gene symbols *FPR1* and *FPRL1*) cross-hybridize with each other and with *FPRL2*, a human gene of unknown expression and function. The *FPR1* product is ~1000-fold more sensitive than the *FPRL1* product to *N*-formylpeptides. We now report cloning of the first cDNA for *FPRL2* and the first description of the RNA distribution in normal human phagocytes for all three genes. *FPR1* and *FPRL1* are expressed in neutrophils and monocytes. In contrast, *FPRL2* RNA is detectable in monocytes but not in neutrophils, and its product could not be activated by *N*-formylpeptides. Thus, the regulation of *FPRL2* gene expression in vivo differs from *FPR1* and *FPRL1*.

N-formylpeptides are products of bacteria that bind to specific receptors on mammalian phagocytes, thereby inducing chemotactic and microbicidal responses (1). The receptor that appears to mediate most if not all phagocyte responses to *N*-formylpeptides is the product of *FPR1*, a human gene for a G protein-coupled receptor designated FPR (2).

Two human genes, designated *FPRL1* and *FPRL2* (L=like), cross-hybridize with FPR DNA probes (3-5). FPR and the FPRL1 receptor (FPRL1R) share 69% amino acid

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identity and bind the prototype ligand *N*-formylmethionyl-leucyl-phenylalanine (fMLP) with high and very low affinity, respectively (2, 3). The putative product of *FPRL2* (also known as *FPRH1*, H = homologue) is 56 and 83% identical to FPR and FPRL1R, respectively, but its ligand has not yet been identified (4). Transcripts for *FPR1* and *FPRL1* have been detected by RNA blot hybridization and by PCR in the transformed myeloid cell lines HL-60 and U937 after treatment with chemical agents that induce differentiation, and their cDNAs have been cloned (2, 3, 5-7). A PCR product reported to be specific for *FPRL2* has been amplified from human lung RNA (6), but an *FPRL2* RNA sequence has not yet been unambiguously demonstrated in any cell type or tissue. To more precisely understand the physiologic regulation of these related genes, we have now defined the number, size, and expression of their transcripts in normal human neutrophils and monocytes.

Materials and Methods

Gene and cDNA Cloning

The *FPRL2* gene was cloned from a human genomic library in the vector Lambda FIX (Stratagene, La Jolla, CA) screened at low stringency using radiolabeled FPR and FPRL1 open reading frame (ORF) probes, using materials and methods previously described (5). A 5 kb *Xba*I fragment of the genomic clone was subcloned into pBluescript and sequenced. A human peripheral blood monocyte cDNA library in a lambda-plasmid vector (8), a gift of S. Gutkind, was screened with a complete, intronless FPRL2 genomic ORF probe at high stringency. Plasmids containing the inserts were produced from the phage DNA by *Not*I digestion and religation as previously described (8).

RNA Analysis

Poly(A)$^+$ RNA was prepared from HL-60 cells incubated for 48 hours with dibutyryl cyclic AMP, a treatment that induces HL-60 cells to differentiate into a neutrophil-like phenotype. Total RNA was also prepared from human peripheral blood neutrophils isolated by Hypaque-Ficoll differential centrifugation, and from human blood monocytes separated from lymphocytes by adherence to plastic for 18 h. RNA was prepared as previously described (5). RNA samples were fractionated by size by denaturing gel electrophoresis, blotted to nitrocellulose or nylon membranes and hybridized to either double-stranded DNA probes labeled with [α-^{32}P]dCTP by the random primer method or to oligonucleotide probes as previously described (5). Antisense oligonucleotides corresponding to the most divergent region of the three open reading frames (ORF), nucleotides 586-625 of the FPRL1 and FPRL2 ORFs, were synthesized, labeled and shown to hybridize specifically with the corresponding cDNA prior to RNA analysis.

Intracellular Calcium Measurements

Granulocytes were isolated from human peripheral blood by Ficoll-Hypaque discontinuous density gradient centrifugation, dextran sedimentation and hypotonic lysis. The granulocytes were typically 95% neutrophils and ~5% eosinophils. Intracellular calcium was measured in neutrophils exactly as previously described (9). Data are presented as the ratio fluorescence of cells loaded with Indo-1 AM (Molecular Probes, Eugene, OR). Data were collected every 200 msec.

Receptor Reconstitution in *Xenopus* Oocytes

The ORFs of *FPRL2* and *FPR1* were amplified by polymerase chain reaction (PCR) using sense primers extending from nucleotide -3 to +18 where nucleotide +1 corresponds to the first nucleotide of the corresponding ORF, and antisense primers corresponding to the final 21 nucleotides of the corresponding ORF. In each case, the choice of the sense primer retained the natural translation initiation sequence. The amplification reaction was performed using 100 ng each of FPR cDNA and a 5 kb *Xba*I genomic fragment of *FPRL2* in pBluescript as templates in a solution containing 2 mM each of dATP, dGTP, dCTP and dTTP, 2.5 units of Taq polymerase and 1x reaction buffer (Boehringer-Mannheim, Indianapolis, IN). The PCR conditions were 94°C for 5 min followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 60 sec. The products were then subcloned into pBluescript and sequenced completely. Capped sense RNA was synthesized by first linearizing plasmid constructs at the 3' end using an appropriate restriction endonuclease that cut in the 3' polylinker region, followed by in vitro transcription with either T3 or T7 RNA polymerase. All cRNA transcripts were shown to migrate as a single appropriately sized band by denaturing agarose gel electrophoresis prior to microinjection. The materials and methods used for the calcium efflux assay were as described (10). Data are presented as the mean ± SEM of the percent of loaded $^{45}Ca^{2+}$ that was released in 20 min by individual oocytes in response to the stimulus. fMLP was from Sigma (St. Louis, MO). fMLP was diluted from a 10 mM stock solution in DMSO into ND96 oocyte media (96 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, pH 7.45).

Results

A single genomic clone was identified for *FPRL2* (GenBank accession number L14061). It contained an intronless ORF encoding a protein that differed from the sequence of Bao et al. (4) by A94G, T231S and H338D, most likely representing allelic variation. Two independent FPRL2 cDNAs were isolated from ~500,000 plaques of a human monocyte library screened with an FPRL2 ORF probe. The longest clone had a 2 kb insert whose sequence extended from nt 171 of the FPRL2 ORF to the end of the ORF, and included 0.9 kb of 3'-UTR.

An FPRL2 ORF probe detects four size classes of HL-60 neutrophil mRNA under low stringency conditions: a tight doublet at ~1.4 kb, and two larger transcripts at 2.6 and 3.5 kb (Fig. 1, lane 1). When the blot is washed under high stringency conditions, the hybrids at 1.4 kb are unstable and melt, whereas the 2.6 and 3.5 kb hybrids are stable (Fig. 1, lane 2). The 1.4 kb doublet corresponds to two distinct FPR mRNAs previously characterized in HL-60 neutrophils by cDNA cloning and primer extension (11). Sequential hybridization of antisense oligonucleotides specific for either FPRL1 or FPRL2 ORFs to the same blot demonstrated that both the 2.6 and the 3.5 kb bands correspond to FPRL1, whereas FPRL2 mRNA is not detectable in HL-60 cells even after prolonged exposures of the blot (Fig. 1, lanes 3 and 4) or by PCR (data not

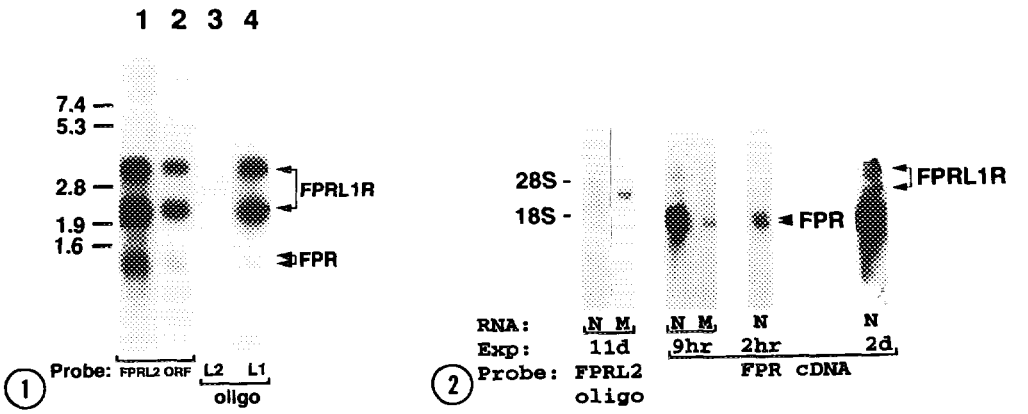

Figure 1. Analysis of *FPR1*, *FPRL1* and *FPRL2* transcripts in HL-60 neutrophils. The same Nytran blot containing 10 μg poly(A)$^+$ RNA from HL-60 neutrophils was hybridized in chronological order with a radiolabeled full-length FPRL2 ORF probe (*lanes 1 and 2*), a specific FPRL2 oligonucleotide probe (*lane 3*), and finally with a specific FPRL1 oligonucleotide probe (*lane 4*). The blot was washed at 55°C in 5x SSPE for 1 h (*lane 1*), at 68°C in 0.1x SSPE for 1 h (*lane 2*), or at 60°C in 5x SSPE for 5 min (*lanes 3 and 4*). The blot was exposed to XAR-2 film with an intensifying screen at -80°C for 6 h (*lane 1*), 2 d (*lane 2*), 7 d (*lane 3*) and 4 d (*lane 4*). The position of chain length RNA standards is indicated in kb at the left.

Figure 2. Analysis of *FPR1*, *FPRL1* and *FPRL2* transcripts in normal human phagocytes. Nitrocellulose blots containing 20 μg of total RNA from neutrophils (*N*) or 3 μg of total RNA from monocytes (*M*) were hybridized with the indicated probes and then washed at high stringency (68°C in 0.5X SSPE for 1 hr, *FPR cDNA*; 58°C in 5X SSPE for 5 min, *FPRL2 oligo*). Washed blots were exposed to XAR-2 film in a Quanta III cassette for the indicated durations. The positions of the twin FPR and FPRL1R-specific transcripts are indicated by arrows. Longer exposures of the monocyte RNA hybridized with the FPR cDNA revealed the cross-hybridizing twin FPRL1R transcripts at 2.6 and 3.5 kb (not shown). Note that all three *N* lanes probed with *FPR cDNA* represent the same experiment exposed for different durations to best reveal the twin FPR and FPRL1R transcripts.

shown). A single class of 3 kb FPRL2 transcripts was detected in monocyte but not in neutrophil RNA with the FPRL2 oligo (Fig. 2). In contrast, two classes each of FPR and FPRL1 specific transcripts could be detected in neutrophil RNA, comparable in size to those found in HL-60 neutrophils. FPR and FPRL1 transcripts were also detected in monocyte RNA.

To address the relative potency of fMLP for calcium mobilization by FPR, FPRL1R and FPRL2, specific cRNA was injected into Xenopus oocytes and acquired ligand-dependent calcium release was measured. The EC_{50} for activation of the calcium efflux response by fMLP for oocytes expressing FPR was ~0.5 nM (Fig. 3B). This value compares favorably with that for elicitation of transient elevations of $[Ca^{++}]_i$ in human neutrophils by fMLP (Fig. 3A). In contrast, the curve was shifted about 1000-

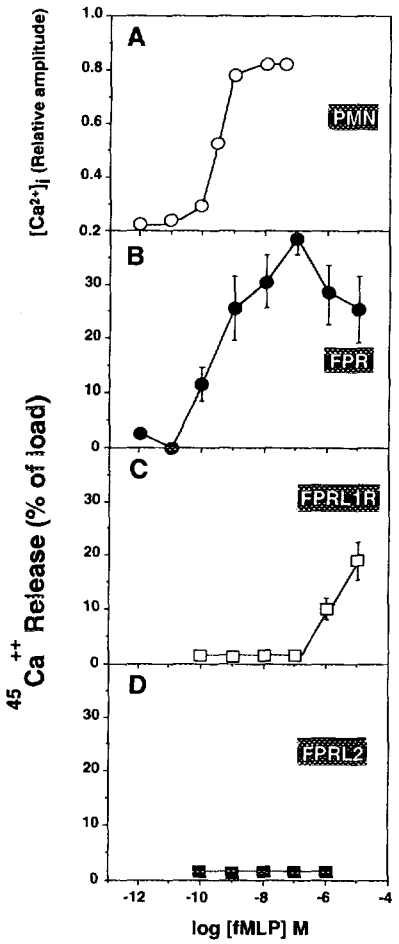

Figure 3. fMLP-induced calcium mobilization in human neutrophils and in frog oocytes injected with FPR, FPRL1R and FPRL2 cRNA. *A*, Neutrophil responsiveness to fMLP. Human neutrophils were loaded with Indo-1 AM and the maximal change in fluorescence was plotted as a function of fMLP concentration. *B-D*, *Xenopus* oocytes were coinjected with 200 ng of HL-60 poly(A)$^+$ RNA (source of a complementary factor previously described [13] that lacks transcripts for FPR, FPRL1R and FPRL2R) and 10 ng of the indicated cRNAs. Four days after injection, oocytes were stimulated with fMLP and calcium efflux was measured. Oocytes that had been injected with 50 nl of water or 200 ng of HL-60 RNA alone did not respond to fMLP over this concentration range (not shown). The data derive from 5-8 replicate determinations per point and are representative of three separate experiments with each receptor. Basal amounts of calcium efflux and calcium uptake were similar for all experimental conditions.

fold to the right in oocytes producing FPRL1R (Fig. 3C), whereas oocytes injected with FPRL2 cRNA did not respond to fMLP (Fig. 3D).

Discussion

Our results unequivocally establish that *FPRL2* is expressed in vivo. FPRL2 mRNA is detectable in normal human monocytes but not in normal human neutrophils, whereas

FPR1 and *FPRL1* are expressed in both cell types, suggesting the involvement of distinct regulatory factors.

Molecular analysis has thus identified potential complexity on the sensing surface of phagocytes that could not have been anticipated based on biochemical and functional studies with fMLP. Despite highly conserved sequences, it appears that FPR alone may account for most if not all of the responses of phagocytes to fMLP. Biochemical characterization of native FPRL1 and FPRL2 receptors awaits the identification of their high affinity ligands. Nevertheless, sequence comparisons and analysis of cloned mammalian FPR and FPR-like receptors with fMLP suggest that residues K85, N185, R267, E279, and D285 of FPR may be particularly important for high affinity binding of fMLP (9, 12). The results of this study will guide future investigations of the transcriptional regulation of the FPR gene cluster, structure-function analysis of FPR, and the search for high affinity ligands for FPRL1R and the FPRL2 product.

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