Cloning of a Human Seven-transmembrane Domain Receptor, LESTR, That Is Highly Expressed in Leukocytes*

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Several chemotactic agonists including interleukin-8 (IL-8) and related cytokines have been shown to activate and attract leukocytes via seven-transmembrane domain, GTP-binding protein-coupled receptors. A cDNA clone, LESTR, encoding a protein of 352 amino acids, corresponding to a novel receptor of this type, was isolated from a human blood monocyte cDNA library. The sequence of the deduced protein, LESTR (leukocyte-derived seven-transmembrane domain receptor), has 92.6% identity with that of a recently reported bovine neuropeptide Y (NPY) receptor, boLCRI (Rilmand, J., Xin, W., Sweetnam, P., Saajoh, K., Nestler, E. J., and Duman, R. S. (1991) Mol. Pharmacol. 40, 869-875). LESTR, however, is more similar (34%) to the IL-8 receptors, IL-8R1 and IL-8R2, and much higher levels of LESTR than IL-8R-specific mRNA were found in human blood neutrophils and lymphocytes. LESTR transcripts, by contrast, were low or undetectable in several neuroblastoma cell lines that are widely used to study NPY functions. Transfected cells expressing high levels of LESTR mRNA did not bind radiolabeled NPY, IL-8, NAP-2, GROα, PF4, IP10, MCP-1, MCP-3, MIP-1α, HC14, 1309, RANTES, C3a, or LTB4. NPY also failed to bind to neutrophils, monocytes, and lymphocytes, to elicit responses in vitro such as Ca2+ changes, shape change, chemotaxis, enzyme release, and the respiratory burst, and to induce leukocyte accumulation upon injection in rats and rabbits. Although the ligand for LESTR could not be identified among a large number of chemotactic cytokines, the high expression in white blood cells and the marked sequence relation to IL-8R1 and IL-8R2 suggest that LESTR may function in the activation of inflammatory cells.

The recruitment of leukocytes in inflamed tissues became a major area of research after the discovery of IL-8 and several related chemotactic cytokines that attract and activate leukocytes (1). These proteins are similar in size, have marked sequence similarities, and are characterized by four conserved cysteines that form two essential disulfide bonds (1). Two subfamilies are distinguished according to the arrangement of the first two cysteines, which are either adjacent (CC subfamily) or separated by one amino acid (CXC subfamily). The CXC cytokines activate primarily neutrophil leukocytes, while CC cytokines act on monocytes (2), basophils (3), and eosinophils (4).

Chemotactic agonists act via seven-transmembrane domain, GTP-binding protein (G-protein)-coupled receptors, as shown originally for fMet-Leu-Phe (5) and C5a (6). Two IL-8 receptors, IL-8R1 and IL-8R2, were characterized subsequently by biochemical, molecular biological, and functional studies (7-9). They bind all known chemotactic cytokines of the CXC subfamily, which are believed also to share the signal transduction system (1). High levels of transcripts for these receptors are found in neutrophils, while expression in other leukocytes is low (10). Chemotactic cytokines of the CC subfamily do not bind to IL-8 receptors. High affinity binding sites for monocyte chemotactic protein 1 (MCP-1) and RANTES have been found on monocytes (11-13).

In view of the similarities among the CXC and CC chemotactic cytokines, we assumed that their receptors are structurally more related to each other than to other G-protein-coupled transmembrane proteins, and we used an IL-8 receptor cDNA probe to isolate putative CC cytokine receptor cDNAs from monocytes. In this paper we describe the isolation of a novel cDNA encoding a seven-transmembrane domain protein, LESTR (leukocyte-derived seven-transmembrane domain receptor), that is closely related to the IL-8 receptors and has more than 90% identity with a bovine brain receptor for neuropeptide Y (NPY), boLCRI (14). Interestingly, this novel human monocyte protein is highly expressed in leukocytes, suggesting that it may be involved in the regulation of their function.

EXPERIMENTAL PROCEDURES

Peptides—Synthetic NPY, IL-8, GROα, GROβ(Y), NAP-2, NAP-2(Y), IP10, PF4, MCP-1, MCP-3, MIP-1α, RANTES, C5α, and HC14 were synthesized and kindly provided by Dr. I. Clark-Lewis, University of British Columbia, Vancouver (15). Bolton-Hunter labeled 125I-NPY was obtained from ANAWA Laboratories, Switzerland or purchased from Amer sham Corp. [3H]LTB4, was purchased from DuPont NEN. C5a was kindly provided by Dr. C. Dahinden, Institute of Clinical Immunology, University of Bern, and 1α,25-dihydroxycholecalciferol (1α,25(OH)2-vitamin D3) by F. Hoffmann-La Roche Ltd., Basel.

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§ The abbreviations used are: IL-8, interleukin 8; NPY, neuropeptide Y; NPYRI, Y1 subtype NPY receptor; hu, human; fMet-Leu-Phe, N-formyl-methionyl-leucyl-phenylalanine; LTB4, leukotriene B4; FCS, fetal calf serum; bp, base pairs; kb, kilobase pairs; TM, transmembrane domain; G-protein, guanine nucleotide-binding regulatory protein; PBL, peripheral blood lymphocytes; COS-7, SV40-transformed monkey kidney cells; CHO-9, Chinese hamster ovary cells; PHA, phytohemagglutinin; 1α,25(OH)2-vitamin D3, 1α,25-dihydroxycholecalciferol.
cells—COS-7 cells were grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM l-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. CHO-9 cells were incubated in Dulbecco's modified Eagle's medium's Ham's F-12 (Amidex), 1:1, supplemented with 10% FCS, 2 mM glutamine, and 30 μg/ml gentamycin. PC12, HEL, SK-N-Be, and 293T/Fc6 cells were obtained from Dr. N. B. Sobieszek, Department of Hematology, University of Bern, and LAN5 and SH-SY-5Y cells from Dr. M. Colombo, National Tumor Institute, Milano. PHA-activated T lymphocyte blasts were prepared by Dr. P. Della Bonna, Basel Institute of Immunology. HL60 cells at 5 x 10^6 cells/ml in RPMI 1640 supplemented with 10% FCS were differentiated with 1.25% dimethyl sulfoxide or with 10 μM 25(OH)D3 vitamin D3 for 72 h. Human neutrophils, monocytes, or peripheral blood lymphocytes (PBL) were prepared from fresh blood or donor blood buffy coats (16, 17).

cDNA Cloning—Total RNA from human monocytes (>95% monocytes, 3–4% lymphocytes) was extracted with the guanidinium thiocyanate/LiCl method (18), and 5 μg of purified poly(A)+ RNA were used for cDNA synthesis (Riboclonc dDNA synthesis system, Promega Corp.). The cDNA fragments were linked to BacI adapters (19), size-fractionated (cut-off size, 1 kb), and ligated into the mammalian expression vector pcDNAI (Inviron Corp.). Finally, Escherichia coli M13 was transfected by electroporation (Bio-Rad), and the monoclonal cDNA libraries of about 4 x 10^8 individual clones with an average insert size of approximately 1.4 kb. For colony-lift hybridization screening (20) about 4 x 10^8 colonies were transferred to Bionyde nylon membranes (PALL AG, Mutzenz) and probed with a 1.919 bp DNA fragment, [32P]P1R (10), coding for part of the human IL-8 cDNA (27). Hybridization was performed in 6 x SSC, 0.05 βtto (20) with 10^6 cpm/ml hybridizing solution at 68 °C for 16 h. The filters were washed once in 2 x SSC, 0.1% SDS at room temperature for 10 min, twice in 1 x SSC, 0.1% SDS at 68 °C for 30 min, and twice in 0.5 x SSC, 0.1% SDS at the same temperature for 15 min. The cDNAs from 60 positive clones were further characterized by restriction enzyme site mapping, Southern hybridization, and partial sequence analysis. Two selected cDNAs of 1.2 and 1.6 kb were subcloned into the Gene Scribe-Z vectors, pTZ18/19/R (U. S. Biochemical Corp.) and sequenced to completion (22). As the nucleotide sequences of both clones were identical within the overlapping region, only the larger cDNA clone, LESTR, was used for further studies.

Northern Analysis—RNA samples of 10 μg were fractionated on denaturing formaldehyde-agarose gels (20), vacuum-transferred (transVAC TE 80, Hoefer Scientific Instruments) onto Nytran membranes (Schleicher and Schuell), and immobilized by baking at 80 °C for 2 h. A 500-bp internal XhoI fragment of LESTR, encoding the second half of the protein, was labeled with [α-32P]CTP by random primed synthesis and used for hybridization at 2 x 10^6 cpm/ml in the presence of 50% formamide at 42 °C for 24 h. The membranes were washed once in 2 x SSC at room temperature for 20 min, two times in 0.25 x SSC, 0.1% SDS, and once in the hybridization solution at 65 °C for 20 min and exposed to Hyperfilm-MP (Amersham International plc) at −70 °C for 1–2 days.

Subcloning and Mammalian Cell Transfection—The complete monoclonal cDNA insert of LESTR was cloned into the SacI site of the pSVL expression vector (Pharmacia). For the generation of CHO transfectants, 4 x 10^4 cells were cotransfected by electroporation with 20 μg of pSVL-LESTR and 10 μg of pVSVωnωn both linearized with EcoRI. Nocodazole-resistant CHO clones were selected by addition of G418 (Life Technologies, Inc.) at 500 μg/ml culture medium and screened for expression of LESTR by Northern blot analysis. Transient transfections in COS cells with pcDNAI-LESTR were performed by the DEAE-dextran method (9) or by electroporation (10).

Receptor Binding Studies—Binding studies were performed with CHO or COS cells expressing LESTR, blood monocytes, neutrophils, and PBL as described (5, 9, 10, 23). [3H]Oxytobin, [3H]NAP-2Y, [3H]CP1, [3H]MCP-1, [3H]-Leukins A, RANTES, IFN, IL-8, HC14, and C3a were iodinated with Enzymobead reagent (Bio-Rad) to a specific radioactivity of 4 x 10^11 dpm/mg. Routinely, cell membranes (10 μg) were incubated with various concentrations of [3H]labeled ligands for 45 min at 4 °C. The membranes were then rapidly filtered through Whatman GF/C filters. They were washed three times with 4 ml of ice-cold binding buffer, placed in 3.5 ml of scintillation mixture, and counted in a liquid scintillation spectrometer.

RESULTS

Isolation of LESTR cDNAs—The monoclonal cDNA library was screened with a polymerase chain reaction probe encoding part of the rabbit IL-8 receptor (10, 21). Out of 60 positive clones, 22 exhibited high hybridization intensities, as shown by Southern blotting. Only one clone for each of the two IL-8 receptors (IL-8Rα and IL-8Rβ) was identified, while all remaining 20 clones shared several common restriction enzyme digestion patterns. Partial sequencing demonstrated that their cDNA was derived from a single RNA species. Two clones of 1201 and 1645 bp were sequenced to completion. Both comprise the whole coding region and have identical nucleotide sequences. The clone with the larger insert (1645 bp), LESTR, was used for detailed characterization (Fig. 1). The longest open reading frame starts with a signal sequence for initiation of translation, is in perfect agreement with the consensus sequence for initiation of translation, and encodes a polypeptide, LESTR, consisting of 352 amino acids with a calculated M, of 39,745. A putative polyadenylation signal, ATTAA, is located 497 bp downstream from the stop codon, TAA, and 16 bp upstream from the poly(A) tail. Sequence analysis revealed that LESTR contains seven puta.
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Fig. 2. Primary structure comparison of LESTR with G-protein-coupled receptors. The multiple protein alignment was performed according to Higgins and Sharp (46) and includes human LESTR, boLCR1, the bovine NPY receptor (14), and huIL-8 receptors type 1 (7) and type 2 (8). The shaded areas designate amino acids that are identical with the corresponding residues in LESTR. Arrowheads indicate potential N-glycosylation sites, and hyphens indicate gaps introduced for optimum alignment. Bars designate the putative transmembrane domains (TM1–TM7).

![Image of protein alignment]

Fig. 3. Northern analysis of LESTR mRNA expression. Upper row, total RNA was extracted from human blood cells and cell lines, namely neutrophils, monocytes, PBL, PHA-activated T cell blasts, HL-60 cells differentiated with 1α,25-(OH)2-vitamin D3, and U937 cells, which express boLCR1 and showed high affinity binding to NPY. Lower row, cDNA was used as hybridizing probe. Lower row, ethidium bromide staining of ribosomal RNA in the gels prior to transfer. M, RNA marker; the band sizes are 0.24, 1.4, 2.4, 4.4, and 7.7 kb.

![Image of Northern blot analysis]
Fig. 4. Northern blot and 125I-NPY binding to CHO transfectants. A, Northern analysis of a selection of neomycin-resistant CHO cell clones cotransfected with pSVL-LESTR and pSV2neo. Each lane was loaded with 10 μg of total RNA, and hybridization was performed as described under "Experimental Procedures." The lower row shows the ethidium bromide staining of ribosomal RNA in the gel prior to transfer. M, RNA marker as in Fig. 3. B, steady-state binding of 125I-NPY to 2D1, a CHO cell transfectant clone expressing LESTR transcripts (upper panel) and SK-N-MC (lower panel). 3 × 10⁶ cells were incubated for 1 h at 37 °C with increasing concentrations of 125I-NPY in the presence (non-specific binding) or absence (total binding) of 1 μM unlabeled NPY. Specifically bound NPY (○, ●) was derived by subtracting the non-specific binding (□, ▲) from the total binding (△, ▲).

nm) for 125I-NPY and low affinity binding for 125I-peptide YY, suggesting that boLCR1 may correspond to the Y₅ subtype of NPY receptors (14). The cDNA for the human NPY Y₁ subtype receptor, huNPYR1, which was recently cloned, conferred high affinity binding (Kᵣ 0.86 nm) for 125I-NPY when expressed in COS cells (31). CHO cells that stably express LESTR, as shown by Northern analysis, were generated by transfection of the corresponding cDNA (Fig. 4A). Binding assays were then performed using cells adherent to plastic or in suspension, as well as membrane preparations.

No specific binding to CHO cell transfectants was observed using 125I-NPY from two different manufacturers in experiments performed according to protocols where incubation time, temperature, and washing procedures were varied systematically. Fig. 4B shows the lack of specific high affinity NPY binding to CHO cell transfectants. Using the same 125I-NPY preparation, 26,000 sites/cell with a Kᵣ of 3 nm were identified on SK-N-MC neuroblastoma cells which bear NPY receptors of the Y₁ subtype (34, 35), demonstrating selective receptor recognition (Fig. 4B). Negative results were also obtained using COS cells that transiently expressed LESTR (not shown).

Since NPY did not bind to cells transfected with LESTR, additional binding studies were performed with human blood phagocytes and lymphocytes that constitutively express high levels of LESTR mRNA (Fig. 3). As in transfected cells, no specific high affinity binding of NPY could be detected on neutrophils, monocytes, or lymphocytes (Table I). The limit of detection, in these experiments, was estimated to be 500 receptors/cell (23).

Functional Assays with Neuropeptide Y—Because of the high level of expression of LESTR in neutrophils (Fig. 3), NPY was tested as potential agonist (Table I). At concentrations between 10⁻⁶ and 10⁻⁹ M, NPY was totally inactive in neutrophils that responded normally to IL-8 in the following functional assays: changes in cytosolic free calcium ([Ca²⁺]i), shape change, chemotaxis, granule exocytosis (following pretreatment with cytochalasin B), and the respiratory burst (1). NPY was also inactive in human blood monocytes and lymphocytes, as indicated by the lack of [Ca²⁺]i changes and chemotaxis in vitro. In addition, exposure of neutrophils and monocytes to NPY (10⁻⁵–10⁻⁶ M for 1, 20, and 40 min) did not affect the time course and extent of the [Ca²⁺]i changes induced by subsequent stimulation with 10⁻⁸ M IL-8 or 10⁻⁷ M monocyte chemotactic peptide 1 (MCP-1) and fMet-Leu-Phe, respectively. Moreover, simultaneous incubation with 10⁻⁷ M NPY had no measurable inhibitory influence on chemotactic activity in neutrophils and monocytes induced with optimal concentrations of IL-8 or MCP-1. Since IL-8 induces neutrophil infiltration when injected into animals (1), NPY was also tested in vivo. It did not induce the infiltration of neutrophils or other inflammatory cells in the skin of rats and rabbits and did not lead to accumulation of leukocytes into the cerebrospinal fluid as tested in a rabbit meningitis model (36).

Binding Studies with Chemokines and Other Chemotactic Agonists—Amino acid sequence comparison shows that LESTR is related more closely to chemotactic receptors in general, and to IL-8 receptors in particular, than to other NPY receptors except for boLCR1 (Fig. 5). These structural similarities and the high expression in leukocytes suggested that LESTR may bind chemotactic cytokines related to IL-8 or MCP-1 (1, 37). Therefore, IL-8 and a number of structurally or functionally related peptides, including GROα, neutrophil-activating peptide 2 (NAP-2), platelet factor 4 (PF4), IP10, MCP-1, MCP-3,
MIP-1α, RANTES, HC14, I309, as well as C3α, were iodinated and tested in binding studies. All ligands used in the binding experiments are listed in Table II. None of them bound specifically to CHO cells expressing LESTR.

**DISCUSSION**

Clones containing fragments of a cDNA encoding a novel putative seven-transmembrane domain receptor, LESTR, were identified in a human monocyte cDNA library by screening with a probe specific for IL-8 receptors. LESTR is highly related to boLCR1, a recently described NPY receptor from bovine brain (14). Human myeloid cells expressed considerably higher levels of LESTR mRNA than transcripts for the two known IL-8 receptors, IL-8Rα and IL-8Rβ (7, 8, 10), as shown by Northern analysis and by the incidence of LESTR clones, which was about 20-fold higher than for IL-8Rα and IL-8Rβ clones in our cDNA library. By contrast, neuroblastoma cell lines that are used to study NPY functions (34, 38) were negative or only weakly positive, except for LAN5 where LESTR expression was high.

The structural relationship to chemoattractant receptors and the high degree of expression in leukocytes suggest that LESTR may be involved in the regulation of leukocyte function. In the present study, however, we found no evidence for binding of NPY on transfected cells as well as human blood monocytes, neutrophils, or lymphocytes, which expressed high levels of LESTR mRNA, and no functional responses were induced in these cells by stimulation with NPY. In addition, NPY also failed to induce leukocyte accumulation and inflammation upon injection in rats and rabbits, indicating that this peptide does not activate inflammatory cells.

NPY exerts a variety of biological effects and has been implicated in the pathophysiology of diseases such as hypertension, congestive heart failure, and behavioral disorders (38). In brain and peripheral nerve tissue, three subtypes of NPY receptors, designated as Y1, Y2, and Y3, are distinguished on the basis of binding and function studies with NPY, peptide YY, and structural analogs (34). Ligand binding to all three receptor types leads to inhibition of adenylyl cyclase. This effect is prevented by pretreatment of the cells with Bordetella pertussis toxin, indicating that signal transduction is mediated by G-proteins of the G1 type (34, 39, 40). Several NPY receptor cDNAs were cloned: huNPYR1 from fetal brain (31) and raNPYR from brain tissue (32) belong to the Y1, raNPYR to the Y2, and boLCR1 to the Y3 type (14). Homology search in the SwissProt data base has shown that the LESTR sequence is only 15–20% identical with huNPYR1, raNPYR, and drNPYR. LESTR and consequently also boLCR1 are more closely related to chemoattractant receptors, including those for fMet-Leu-Phe, C5α, and platelet-activating factor, and share an overall sequence identity of 34% with IL-8 receptors (7, 8, 30). Despite
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this sequence similarity, CHO cells transfected with LESTR did not bind the chemotactants IL-8, GROα, NAP-2, PF4, IP10, MCP-1, MCP-3, MIP-1α, RANTES, HC14, I306, C3α, and LTB4.

Recent Southern experiments with human genomic DNA under high stringency conditions using an internal XhoII fragment of LESTR revealed a hybridization pattern that corresponded to the presence of a single LESTR gene. Additional signals could not be detected, suggesting that the LESTR gene has no other homologues in the human genome and thus appears to be the most closely related homologue to boLCR1.

It has been reported that boLCR1, like huNPYR1, binds NPY with high affinity although the sequence identity between these two receptors is only 18%. In view of these data, it was surprising to realize that LESTR, which appears to be the most closely related homologue to boLCR1. It may be used to establish the structural requirements of a total of 11 non-conservative amino acid substitutions are considered, did not bind NPY. Few amino acid substitutions may suffice to alter the ligand specificity. Of a total of 11 non-conservative amino acid substitutions in LESTR with respect to boLCR1, seven are in the extracellular loops, one in TM4 and one in the N-terminal proximity of TM1. Site-directed mutagenesis of LESTR and boLCR1 may be used to establish the structural requirements for NPY binding.

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