Molecular Cloning and Functional Characterization of a Novel Human CC Chemokine Receptor (CCR5) for RANTES, MIP-1β, and MIP-1α*

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Chemokines affect leukocyte chemotactic and activation activities through specific G protein-coupled receptor genes. In an effort to map the closely linked CC chemokine receptor genes, we identified a novel chemokine receptor encoded 18 kilobase pairs downstream of the monocytic chemoattractant protein-1 (MCP-1) receptor (CCR2) gene on human chromosome 3p21. The deduced amino acid sequence of this novel receptor, designated CCR5, is most similar to CCR2B, sharing 71% identical residues. Transfected cells expressing the receptor bind RANTES (regulated on activation normal T cell expressed), MIP-1β, and MIP-1α with high affinity and generate inositol phosphates in response to these chemokines. This same combination of chemokines has recently been shown to potently inhibit human immunodeficiency virus replication in human peripheral blood leukocytes (Cocchi, F., DeVico, A. L., Garzino-Demo, A., Arya, S. K., Gallo, R. C., and Lusso, P. (1995) Science 270, 1811-1815). CCR5 is expressed in lymphoid organs such as thymus and spleen, as well as in peripheral blood leukocytes, including macrophages and T cells, and is the first example of a human chemokine receptor that signals in response to MIP-1β.

Chemokines mediate the migration and activation of leukocytes at sites of inflammation. Chemokines are 70–90 amino acids in length and are subdivided into two gene families based on the presence or absence of an amino acid between the first two of four conserved cysteines (1, 2). The CXC chemokines predominantly activate neutrophils, while the CC chemokines generally activate monocytes, lymphocytes, basophils, and eosinophils.

Leukocytes respond to chemokines through specific cell surface receptors, all of which are members of the G protein-coupled receptor superfamily. The first chemokine receptor to be identified are expressed on neutrophils and recognize CXC chemokines; interleukin-8 receptor A (IL8RA)1 recognizes only IL-8 (3), while IL8RB recognizes IL-8, melanoma growth stimulatory activity (GROMGSA), and neutrophil-activating protein-2 (NAP-2) (4). A promiscuous chemokine receptor, the Duffy blood group antigen, has been characterized from erythrocytes and recognizes numerous CXC and CC chemokines (5, 6). More recently, four human CC chemokine receptors (CCRs) have been characterized. CCR1 is specific for MIP-1α, RANTES, and MCP-3 (7–9); CCR2A and CCR2B (first identified as the MCP-1 receptors) are alternatively spliced gene products which recognize both MCP-1 and MCP-3 (10–12); CCR3 is expressed on eosinophils and recognizes eotaxin (13, 14); and CCR4 is found on basophils and responds to MIP-1α, RANTES, and MCP-1 (15).

Genes encoding closely related G protein-coupled receptors are often closely linked. For example, the IL-8 receptor genes (IL8RA, IL8RB, and a related pseudogene) are all encoded by human chromosome 2q34-q35 (16). In addition, the receptor genes for chemoattractants C5a and fMet-Leu-Phe as well as two closely related orphan receptor genes cluster at chromosome 19q13.3 (17). Recently, the genes for CCR1, CCR2, and the related sequence V28 (18) have been mapped to human chromosome 3p21 (8, 18–20). In our efforts to further analyze the genetic linkage of these CC chemokine receptor genes, we have identified a novel G protein-coupled receptor on chromosome 3 with significant homology to this gene family. This receptor binds and functionally responds to a unique combination of CC chemokines and is therefore termed CCR5.

EXPERIMENTAL PROCEDURES

Materials and Chemicals—The chemokines MCP-1, MIP-1α, MIP-1β, RANTES, and IL-8 were obtained from R&D Systems, Inc. (Minneapolis, MN). LipofectAMINE, Opti-MEM, Dulbecco's modified Eagle's medium, and modified Eagle's medium with Earle's balanced salts were obtained from Life Technologies, Inc. Fetal calf serum was obtained from Hyclone Laboratories, Inc. (Logan, UT). myo[2-3H]inositol was obtained from DuPont NEN. The chimeric G protein Gq5 expression plasmid (21) was a generous gift of Dr. Bruce Conklin (Gladstone Institute of Cardiovascular Disease). Plasmid pBJ1 was from Dr. Mark Davis (Stanford University).

Screening YAC Library—Two yeast artificial chromosome (YAC) clones encoding CCR1 were identified by PCR on DNA pools from the human CEPH "B" YAC library (Research Genetics Inc., Huntsville, AL). DNA from YAC clones 881F10 and 941A7 were used as templates for PCR reactions with degenerate primers designed from homologous regions of CCR1, CCR2, and V28 (18). The primers corresponded to regions in the second intracellular loop and the sixth transmembrane domain of the receptor proteins: sense, GACGGATCCAT(T/C)GA(T/G)TGGAT(A/T)GGA(A/G)AG(A/T)CA(T/G)G(T/C)G and C(T/G)G(T/C)GGATCCAT(T/C)GA(T/G)TGGAT(A/T)GGA(A/G)AG(A/T)CA(T/G)G(T/C)G.

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The abbreviations used are: IL8R, interleukin-8 receptor; IL-1α, interleukin-1α; CCR, CC chemokine receptor; MCP, monocyte chemoattractant protein-1; MIP, macrophage inflammatory protein; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RANTES, regulated on activation normal T cell expressed; YAC, yeast artificial chromosome; kb, kilobase(s).
The PCR reactions were performed using a single 4-min denaturing step at 94°C followed by 33 cycles of denaturing for 1 min at 94°C, annealing for 45 s at 55°C, and extension for 1 min at 72°C. The resulting PCR products were digested with BamHI (site underlined in sense primer) and HindIII (site underlined in antisense primer) and cloned into pBluescript (Stratagene, La Jolla, CA) for sequence analysis.

Isolation of CCR5 cDNA—A human macrophage cDNA plasmid library (22) in pRcCMV (Invitrogen, San Diego, CA) was screened by PCR with primers specific for CCR5 (sense, TGTGTTTGCTTTAAAAGCC; antisense, TAAGCCTCACAGCCCTGTG). The PCR reactions were performed using a single 4-min denaturing step at 94 °C followed by 33 cycles of denaturing for 1 min at 94 °C, annealing for 45 s at 55 °C, and extension for 1 min at 72 °C. The resulting PCR products were digested with BamHI (site underlined in sense primer) and HindIII (site underlined in antisense primer) and cloned into pBluescript (Stratagene, La Jolla, CA) for sequence analysis.

RACE PCR—RACE PCR was performed on human spleen 5'-RACE-ready cDNA (Clontech, Palo Alto, CA) using antisense primers specific for CCR5 according to the manufacturer’s directions. The resulting PCR product was ligated into vector pCR using a TA cloning kit (Invitrogen) and sequenced.

Mapping CCR Locus—Three P1 clones (Genome Systems, St. Louis, MO) were identified using PCR primers specific for CCR2. Genomic mapping was performed by Southern blotting of restriction endonuclease digests of P1 and YAC DNA. Restriction fragments were separated by pulse field or conventional electrophoresis, transferred to nylon membranes, and hybridized with 32P-labeled DNA probes from different regions of the CCR genes.

Northern Blots—The expression of CCR5 mRNA in human tissues was examined by Northern analysis using blots purchased from Clontech. The blots were hybridized in 0.75 M sodium chloride, 50 mM sodium phosphate (pH 6.8), 5 mM EDTA, 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 100 μg/ml sheared salmon sperm DNA, 2% sodium dodecyl sulfate, and 50% formamide at 42 °C overnight using a partial CCR5 cDNA as probe (including approximately 700 bases of coding region and 300 bases of 3' non-coding). Blots were washed extensively at 50 °C in 30 mM sodium chloride, 3 mM sodium citrate, 0.1% sodium dodecyl sulfate.

Fig. 1. CCR5 cDNA sequence. The CCR5 cDNA sequence was compiled from macrophage cDNA clones 134 and 101 and from data obtained by RACE PCR. The deduced amino acid sequence is shown below the DNA sequence.
CD4⁺ and CDB⁺ T lymphocytes were isolated from normal human blood by immunomagnetic negative selection. Purity of each T cell preparation was >90%. RNA was isolated from T cells and from hematopoietic cell lines using RNA STAT-60 (Tel-Test B, Friendswood, TX). Ten µg of total RNA from each cell type was reverse transcribed into cDNA using Superscript II reverse transcriptase (Gibco BRL). The CCR5 coding region was subcloned into the mammalian cell expression vector pBJ1, which is derived from pCIneo. The half of the CCR5 coding region was used as a probe.

Expression of CCR5—The CCR5 coding region was subcloned into the mammalian cell expression vector pCIneo, which is derived from pcDL-SRα (23). This construct contains a signal sequence followed by the FLAG epitope. pcDL-SRα was transfected into HEK-293 cells using LipofectAMINE according to the manufacturer's instructions. Cells were expanded in the presence of G418 (800 µg/ml). Transfected cells were expanded in the presence of G418 (800 µg/ml). Transfected cells were transfected into HEK-293 cells using LipofectAMINE following the manufacturer's instructions. For binding studies, the FLAG-CCR5 sequence was subcloned into pcDNA3 (Invitrogen) and transfected into HEK-293 cells using LipofectAMINE according to the manufacturer's instructions. Cells were expanded in the presence of G418 (800 µg/ml). Transfected cells were evaluated for expression of CCR5 at the cell surface by enzyme-linked immunosorbent assay using the M1 antibody (Eastman Kodak Co.) to the FLAG epitope.

Phosphoinositol Hydrolysis—Transfected cells were assayed for agonist-dependent phosphoinositol turnover as described (25). Briefly, approximately 24 h after transfection, COS-7 cells were labeled with [3H]inositol (100 µCi/ml) in isositol-free medium containing 10% dialyzed fetal calf serum. Labeled cells were washed and then treated with agonist for 1 h at 37 °C in isositol-free Dulbecco's modified Eagle's medium containing 10 mM LiCl. Cells were lysed by addition of 0.75 ml of ice-cold 20 mM formic acid for 30 min. Supernatant fractions were loaded onto AG1-X8 Dowex columns (Bio-Rad) followed by immediate addition of 3 ml of 50 mM NH₄OH. The column was then washed with 4 ml of 40 mM ammonium formate followed by elution with 2 M ammonium formate. Total inositol phosphates were quantitated by counting β emissions.

Binding Assay—The radiolabeled chemokine binding assay was a modification of the procedure described by Ernst et al. (26). Chemokines were labeled by the Bolton and Hunter reagent (diiodo, DuPont NEN) according to the manufacturer's instructions. Unconjugated iodide was separated from labeled protein using a PD-10 column (Pharmacia Biotech Inc.) equilibrated with phosphate-buffered saline and bovine serum albumin (1% w/v). The specific activity was typically 2200 Ci/mmol. Equilibrium binding was performed by adding [3H]-labeled hMIP-β with or without a 100-fold excess of unlabeled ligand to 5 × 10⁵ cells in a total volume of 300 µl of binding buffer (50 mM Hepes pH 7.4, 1 mM CaCl₂, MgCl₂, 0.5% bovine serum albumin) and incubating for 90 min at 27 °C with shaking at 150 rpm. The cells were collected using a Skatron cell harvester (Skatron Instruments Inc., Sterling, VA) on glass fiber filters presoaked in 0.3% polyethyleneimine and 0.2% bovine serum albumin. Bound ligand was quantitated by counting β emissions. Competitive binding was determined by incubation of 5 × 10⁵ transfected cells (as above) with 2 nM radiolabeled hMIP-β and the indicated concentrations of unlabeled ligand. The data were analyzed using the curve-fitting program Prism (GraphPad Inc., San Diego, CA) and the iterative nonlinear regression program LIGAND (27).

RESULTS AND DISCUSSION

Previously characterized CC chemokine receptors are structurally similar, sharing 46–62% amino acid identity (19). In addition, CCR1, CCR2, and the closely related G protein-coupled receptor V28 are known to be encoded by human chromosome 3p21 (8, 18–20). The close proximity of these CC chemokine receptor genes suggested that related genes might be clustered nearby. Two overlapping human YAC clones were identified by PCR with primers specific for the CCR1 gene. The clones, 881F10 and 941A7, were 640 and 700 kb, respectively, and both mapped to human chromosome 3 (28, 29). Amplification with primers specific for one of the CC chemokine receptor genes demonstrated that both YAC clones also encoded CCR2 and the closely related G protein-coupled receptor V28.

To look for novel receptor genes, YAC DNA was used as a template for PCR with degenerate oligonucleotides. The beginning of the second cytoplasmic domain and the sixth transmembrane domain, which are highly conserved among CCR1, CCR2, and V28, were used to design the degenerate oligonucleotides for PCR. In addition to CCR1 and CCR2, two novel G protein-coupled receptor genes were amplified. One of the genes, 88B-28, was subsequently reported to be the eotaxin receptor gene CCR3 (13, 14, 19). The other novel sequence was utilized to isolate two full length cDNA clones from a macro-

Comparison of CCR protein sequences. The deduced amino acid sequence of CCR5 with other CCRs. Putative transmembrane domains are overlined and residues found at the same position in all five receptors are shaded in gray.
phage cDNA library. Both cDNA clones have an open reading frame of 352 amino acids that contains features of a seven-
transmembrane receptor (Fig. 1). Clone 134 is 1.6 kb in length
and extends 45 bases upstream of the putative initiating me-
thionine. Clone 101 is 3.4 kb in length, extends 25 bases up-
stream of the initiating methionine, and includes a poly(A) tail.
A consensus Kozak sequence (30) surrounds the putative ini-
tiating methionine codon; however, neither clone contains an
in-frame stop codon in the 5'-untranslated region. To confirm
that the putative initiating methionine is the true translational
start, RACE PCR was performed on human spleen cDNA. The
fragment amplified by RACE PCR extends 9 nucleotides far-
ther upstream than clone 134; however, these nucleotides do
not encode another methionine or a termination codon. There-
fore, the originally designated initiating methionine is as-
sumed to be correct.

The deduced amino acid sequence of the novel gene shares
significant sequence identity with all four of the known CC
chemokine receptors (Fig. 2). It is most similar to CCR2B with
71% identical residues and shares 55, 49, and 48% identity
with CCR1, CCR3, and CCR4, respectively. The sequences
diverge significantly in their amino-terminal extracellular do-
 mains, a region that has been implicated in determining ligand
specificity (31). As shown below, the receptor encoded by this
novel sequence binds and responds to a unique set of CC
chemokines and is therefore termed CCR5.

The position of CCR5 was mapped relative to the other CC
chemokine receptor genes at this locus on chromosome 3p21.
As shown in Fig. 3B, four receptor genes are closely linked,
mapping within approximately 150 kb of each other as deter-
mined by pulse field electrophoresis and Southern blotting of
YAC clones. The fifth CC chemokine receptor gene, CCR4 (15),
was not found on either of the YAC clones. Significantly, CCR5
maps within 18 kb of CCR2, the gene to which it is most
similar. Overlapping P1 clones were used to restriction map
and define the intron/exon structure of these two closely re-
lated genes (Fig. 3B). CCR5 contains a single intron of 1.9 kb

![Fig. 3. Map of CCR gene locus.](image)

![Fig. 4. Tissue distribution of CCR5 expression.](image)
between nucleotides -11 and -12 in the 5'-untranslated region of the cDNA. CCR2 contains at least two introns, an alternately spliced 1.2-kb intron in the coding region and an intron greater than 2.7 kb that interrupts the 5'-untranslated region between nucleotides -51 and -52 of the cDNA.

The expression of CCR5 mRNA in human tissues was examined by Northern blot (Fig. 4A). A transcript of approximately 3.5 kb was found at highest levels in thymus and spleen, at medium levels in peripheral blood leukocytes and small intestine, and at low levels in ovary and lung. CCR5 expression in hematopoietic cell lines and in human T lymphocytes was also determined by Northern blot analysis (Fig. 4B). The transcript is present at highest levels in the myeloid cell line THP-1 and in CD4+ and CD8+ T cells. It was also detectable at lower levels in the myeloid cell line HL-60, in the B cell line Jijoye, and in the T cell line HUT 78. In addition, the cDNA was an abundant transcript in our human macrophage cDNA library.

CCR5 was transfected into COS-7 cells for intracellular signaling studies to determine ligand specificity. The FLAG epitope (DYKDDDDK) was added to the amino terminus of CCR5 to facilitate detection of receptor expression (24). Previous experiments using CCR2B have shown that addition of this epitope does not affect ligand binding or receptor signaling and may increase surface expression. Quantitative enzyme-linked immunosorbent assays confirmed that CCR5 was expressed at the cell surface in transiently transfected COS-7 cells; however, no phosphoinositid hydrolysis was detected in response to CC or CXC chemokines (data not shown). Other laboratories have shown that some chemokine receptors such as IL8RA and IL8RB require cotransfection with exogenous G proteins before signaling can be detected in COS-7 cells (32). To optimize signaling through CCR5 in the COS-7 cells, the receptor was co-expressed with the chimeric G protein Gi5 (in which the carboxyl-terminal five amino acids of Gq2, which mediate receptor binding, replace those of Goq; see Ref. 21). Previous results have shown that Gi5 significantly potentiates signaling by CCR1 and CCR2B.2 Co-transfection of CCR5 with Gi5 revealed that CCR5 signaled well in response to RANTES, hMIP-1β, and hMIP-1α in phosphoinositid hydrolysis assays (Fig. 5A). Murine chemokines MIP-1α and MIP-1β also stimulated inositol phosphate release in transfected cells. No signaling was measured in response to hMCP-1, IL-8, or the murine MCP-1 homologue JE. Dose-response curves indicated EC50 values of 1 nM for RANTES, 6 nM for hMIP-1β, and 22 nM for hMIP-1α (Fig. 5B). The murine homolog of CCR5 has recently been isolated and recognizes a similar set of ligands (33).

Binding of radiolabeled hMIP-1β to 293 cells expressing CCR5 was examined. Equilibrium binding experiments showed that hMIP-1β bound CCR5 transfected cells in a specific and

2 Montedaro, F. S., and Charo, I. F. (1996) J. Biol. Chem. 271, in press.

3 Arai, H., and Charo, I. F. (1996) J. Biol. Chem. 271, in press.
saturable manner (Fig. 6A). Scatchard analysis of the binding data revealed a dissociation constant ($K_d$) of 1.6 nM and an average number of sites per cell of $1.2 \times 10^5$ (data not shown). Competition of hMIP-1β binding was observed with hMIP-1β, hMIP-1α, and RANTES (Fig. 6B). IC$_{50}$ values obtained from competition binding curves were 6.9 nM for RANTES, 7.4 nM for MIP-1α, and 7.4 nM for MIP-1β. Interestingly, the affinity of MIP-1α binding to CCR5 is as high as the other ligands, even though it is less potent at inducing inositol phosphate release. This disparity between binding and signaling potency has also been observed with MIP-1α and RANTES interactions with CCR1 (7).

CCR5 is the first cloned human receptor that responds to MIP-1β. Although Combadiere and colleagues originally reported that CCR3 signaled in response to MIP-1β (34), they subsequently reported that this response was not due to CCR3 (35). In contrast to MIP-1α, relatively little is known of potential biological roles for MIP-1β. Sporn and colleagues have demonstrated an increase in MIP-1β mRNA following monocyte adhesion to substrates (36). Evidence for a role in mediating lymphocyte migration was provided by Tanaka and colleagues, who found that immobilized MIP-1β induced activation and adhesion of CD8$^+$ T cells to vascular cell adhesion molecule (VCAM) (37). It is intriguing that the same three chemokines that activate CCR5 have recently been shown by Cocchi and colleagues (38) to potently inhibit replication of human immunodeficiency viruses types 1 and 2 in human peripheral blood leukocytes. This raises the possibility that activation of CCR5, which is expressed in T lymphocytes and macrophages, may play a protective role in human immunodeficiency virus infection. The availability of the CCR5 cDNA represents an important tool for elucidating the roles of MIP-1β and related chemokines in lymphocyte activation, trafficking, and human immunodeficiency virus infection.

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Note Added in Proof—Samson et al. (39) recently reported the genomic sequence of CCR5.