CCR11 Is a Functional Receptor for the Monocyte Chemoattractant Protein Family of Chemokines*

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Chemokines mediate their diverse activities through G protein-coupled receptors. The human homolog of the bovine orphan receptor PPR1 shares significant similarity to chemokine receptors. Transfection of this receptor into murine L1.2 cells resulted in responsiveness to monocyte chemoattractant protein (MCP)-4, MCP-2, and MCP-1 in chemotaxis assays. Binding studies with radiolabeled MCP-4 demonstrated a single high affinity binding site with an IC50 of 0.14 nM. As shown by competition binding, other members of the MCP family also recognized this receptor. MCP-2 was the next most potent ligand, with an IC50 of 0.45 nM. Surprisingly, eotaxin (IC50 = 6.7 nM) and MCP-3 (IC50 = 4.1 nM) bind with greater affinity than MCP-1 (IC50 = 10.7 nM) but only act as agonists in chemotaxis assays at 100-fold higher concentrations. Because of the high affinity binding and functional chemotactic responses, we have termed this receptor CCR11. The gene for CCR11 was localized to human chromosome 3q22, which is distinct from most CC chemokine receptor genes at 3p21. Northern blot hybridization was used to identify CCR11 expression in heart, small intestine, and lung. Thus CCR11 shares functional similarity to CCR2 because it recognizes members of the MCP family, but CCR11 has a distinct expression pattern.

Chemokines are a family of small proteins, usually 70–90 amino acids in length, that are responsible for the directed migration of specific cell types (for reviews, see Refs. 1–6). The complexity and functions of the chemokine family, now with more than 30 genes, have become increasingly diverse as more members have been identified and characterized. Chemokines play a critical role in the host response to infection because they are responsible for recruitment of leukocyte subsets to sites of pathogen entry (7, 8). Many inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease, and asthma (9), have been associated with elevated chemokine expression. In addition, chemokines are also responsible for the migration of cells within certain lymphoid organs that are critical for leukocyte development, such as thymus (10–12), lymph node (13), and spleen (14, 15). As shown by gene targeting studies, the chemokine stromal cell-derived factor (SDF)-1 is critical for proper neuronal and cardiac development (16, 17). Chemokines have also been implicated in cardiovascular processes such as angio- genesis and atherosclerosis (18).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AF193507.

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EXPERIMENTAL PROCEDURES

Materials and Reagents—The chemokines IL-8, IP-10, I-309, SDF-1, MIP-1α, MIP-1β, MCP-1, MCP-3, MCP-4, ELC (also known as MIP-3β), SLC (6CKine), NAP-2, ENA-78, HCC-4, HCC-1, LKN-1 (MIP-5 or HCC-2), lymphotactin, and fractalkine were purchased from R & D Systems (Minneapolis). MCP-1, PARC, MDC, TARC, and eotaxin were purchased from Gyrphyn Sciences (San Francisco, CA). PF-4, MCP-2, MGS, MIG, RANTES, TECK, and LARC (MIP-3β) were purchased from Peprotech (Rocky Hill, NJ).

Isolation of CCR11 cDNA and Gene—The GenBank Expressed Sequence Tag (EST) data base was searched with the bovine PPR1 cDNA sequence (21) using the BLAST algorithm (22). Three human ESTs were identified (H67224, AA15577, AI15555) with high homology to the bovine sequence. The clone H67224 was obtained from Research Genetics (Huntsville, AL), and the entire insert was sequenced. Because this EST contained only a fragment of the coding region, additional cDNA libraries were screened. Three human cDNA libraries were hybridized with a probe from the EST sequence (prepared by polymerase chain reaction amplification with the primers 5′-GCTTCTGGAAATGCAGTTTCTGG and 5′-CGATGTCATCGTGGTTGTCTCA: small intes- trine (Strategene, La Jolla, CA), macrophage (described by 23), and

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Peripheral blood mononuclear cell (phorbol myristate acetate/ionomycin-stimulated, 24). More than a million clones were examined in each library. No clones were found in the macrophage library. A single clone was identified in the peripheral blood mononuclear cell library which was 1388 bp in length and lacked 188 bp of the amino-terminal coding sequence. These clones were isolated from the small 5.5-kb lambda library, ranging in size from 131 to 1153 bp. The consensus cDNA sequence was missing 14 bp from the 5′-end of the coding region when aligned with the bovine PPR1 coding region sequence. To determine the amino-terminal coding region, a human genomic P1 library (Genome Systems Inc., St. Louis) was screened by polymerase chain reaction with the above clones to isolate the CCR11 gene. The 5′-end of the isolated clone was sequenced with primers based on the cDNA sequence. The deduced genomic sequence provided the remaining coding sequence for CCR11. The genomic sequence presented in Fig. 1 (residues 1–275) appears to contain no intervening sequences because it has contiguous homology with the bovine cDNA sequence (21). Four nucleotide differences were identified, one of which resulted in an amino acid change at position 143 (lysine, in the genomic and small intestine clones, to asparagine, in the peripheral blood mononuclear cell clone).

**RESULTS**

**Isolation of the Human Gene for PPR1—**Matsuoka and colleagues (21) previously isolated an orphan GPCR from bovine taste papillary tissue. Hydropathy and sequence analyses demonstrated that PPR1 was a member of the GPCR superfamily. More recent homology comparisons suggested a closer relationship to chemokine receptors than gustatory or olfactory receptors. Three human EST cDNA sequences were identified in the GenBank data base with high homology to the bovine PPR1 sequence. Oligonucleotide primers were designed from the human sequences and used to identify six partial cDNA clones and a genomic P1 clone of approximately 90 kilobase pairs. The genomic P1 clone was used to isolate the entire coding sequence. Based on the functional sequence data below, we have designated this human gene CCR11.

The CCR11 DNA sequence and encoded amino acid sequence are presented in Fig. 1. Hydropathy analysis (not shown) delineated seven hydrophobic domains typical of a seven-transmembrane spanning GPCR. Human CCR11 is 86% identical to bovine PPR1 at the amino acid level. This high degree of similarity is consistent with other GPCR genes when compared across mammalian species. Like most GPCRs, CCR11 contains preferential evolutionary relationship to chemokine receptors than gustatory or olfactory receptors. Three human EST cDNA sequences were identified in the GenBank data base with high homology to the bovine PPR1 sequence. Oligonucleotide primers were designed from the human sequences and used to identify six partial cDNA clones and a genomic P1 clone of approximately 90 kilobase pairs. The genomic P1 clone was used to isolate the entire coding sequence. Based on the functional sequence data below, we have designated this human gene CCR11.

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centromere to the telomere of chromosome arm 3q, an area that corresponds to 3q22.

Northern Blot Analysis—To determine sites of expression of CCR11, Northern blot hybridizations were performed. The CCR11 gene was used as a hybridization probe for 12 different human tissues. CCR11 was expressed most abundantly in human heart, small intestine, and lung (Fig. 4). Lower levels of hybridization were observed in kidney, liver, and colon. The size of the primary transcript is approximately 2000 bases, which corresponds well with the cDNA size. The most abundant transcript in heart appears to be of greater size than that seen in other tissues and perhaps represents an alternatively spliced transcript.

Functional Responses of CCR11 Transfectants—Murine L1.2 cells were transfected with CCR11 and then tested for chemotaxis to a panel of 29 human chemokines. This panel included MIP-1α, MIP-1β, RANTES, MCP-1, MCP-2, MCP-3, MCP-4, eotaxin, ELC, SLC, LARC, PARC, MDC, TARC, TECK, IL-8, IP-10, I-309, SDF-1, MGSA, MIG, NAP-2, ENA-78, PF-4, HCC-1, HCC-4, LKN-1, lymphotactin, and fractalkine. Each chemokine was tested at 1 nM and 10 nM because these concentrations are generally optimal for other chemokine-chemokine receptor combinations. The most significant migration was observed to MCP-4, with some chemotaxis also observed toward MCP-2 and MCP-1. No other chemokines induced significant cell migration. The CCR11 transfectants that migrated toward MCP-4 were harvested, cloned by limiting dilution, and expanded for further functional studies. As shown in Fig. 5, CCR11 transfectants selected in this manner were tested in chemotaxis assays with a range of concentrations of MCP-4,
MCP-1, MCP-2, MCP-3, and eotaxin. Confirming the original observation, CCR11 transfectedants migrated most efficiently toward MCP-4, with peak chemotaxis occurring at 10 nM. Significant migration was also observed toward MCP-2 and MCP-1 with peak chemotaxis occurring at 10–100 nM, although the number of cells migrating was slightly less than MCP-4. MCP-3 and eotaxin functioned as agonists only at the highest concentration of 1 μM.

CCR11 transfectants were also tested for calcium mobilization in response to ligand stimulation. Small but significant calcium flux was observed when transfectants were stimulated with MCP-4 (results not shown). This response was quantitatively not as strong as we have observed previously with other chemokine receptor-ligand pairs (see “Discussion”). No significant calcium flux was observed in response to MCP-1 or MCP-2 stimulation.

Receptor Binding Assays—Because MCP-4 was the most potent functional ligand, radiolabeled MCP-4 was used as a probe to examine binding to CCR11 transfected L1.2 cells. As shown in Fig. 6A, the 125I-MCP-4 binding was inhibited competitively with increasing concentrations of unlabeled MCP-4 (IC50 of 0.140 nM), MCP-2 (IC50 of 0.458 nM), MCP-3 (IC50 of 4.08 nM), eotaxin (IC50 of 6.72 nM), or MCP-1 (IC50 of 10.7 nM). This suggests that all five ligands recognize a common binding site on CCR11 and that MCP-4 exhibits the greatest affinity. The observed binding of MCP-4, MCP-2, and MCP-1 is consistent with the functional chemotactic responses described above. However, MCP-3 and eotaxin bind with reasonable affinity but only act as agonists at more than 100-fold higher concentrations.

To examine specificity of binding to CCR11, 17 additional chemokines were tested at 1000-fold molar excess for competition of radiolabeled MCP-4 binding. The MCP family members, including eotaxin, effectively competed with 125I-MCP-4 for binding to CCR11 (Fig. 6B). The other chemokines did not compete for CCR11 binding even at this high concentration.

**DISCUSSION**

CCR11 was identified during a search of the human EST data base for homologs of the bovine orphan PPR1. When the full coding region of CCR11 was assembled, it was found to be 86% identical to PPR1 at the amino acid level. Homology com-
Comparisons indicated that CCR11 is most closely related to chemokine receptors. Its closest relatives are CCR7 (36% identical), CCR6 (33%), and CCR9 (33%). Chromosomal mapping of CCR11 localized it to 3q22. Interestingly, many other CC chemokine receptors also map to chromosome 3, including CCR1, CCR2, CCR3, CCR4, CCR5, and CCR8 (26). CCR11, however, is significantly separated from these receptors, which are clustered at 3p21–24. This suggests that CCR11 is more distantly related to most CC chemokine receptors, consistent with the sequence homology comparisons presented in Fig. 2. The CCR11 gene maps somewhat closer to the orphan receptor GPR15 (27; also known as BOB, 28) which is located at 3q11.2–13.1 (27).

As demonstrated in binding and chemotaxis studies, CCR11 is a chemokine receptor that recognizes ligands in the MCP family. The primary ligands for CCR11 are MCP-4 and MCP-2, based on binding affinities and agonist properties in chemotaxis experiments. Other MCP family members also interact with CCR11 with lower affinities. Although CCR11 is most closely related to CCR7, it does not interact with the CCR7 ligands ELC and SLC.

The MCPs share high homology with each other (56–72%) and form their own branch of the CC chemokine family tree. In addition, the MCPs share some functional similarity and are all closely linked on human chromosome 17q11.2 (29). However, MCP expression patterns are distinct, with MCP-4 being expressed constitutively in lung, small intestine, and colon (30, 31), whereas MCP-1 is expressed primarily in cells stimulated with proinflammatory agents (32, 33). MCP-4 has been identified previously as an agonist for CCR2 and CCR3 (30, 31). MCP-2 is recognized by CCR1, CCR2, CCR5, and CCR5 (34–36), MCP-1 is the strongest ligand for the receptor CCR2 (37), and this receptor also recognizes MCP-2 (34), MCP-3 (38), and MCP-4 (30, 31). The characterization of MCP family members as ligands for CCR11 adds additional complexity and redundancy to this diverse repertoire of chemokine functions.

Identification of ligands for orphan GPCRs can be complex. GPCRs can exhibit paradoxical behavior, particularly transfected recombinant receptors. Although not well understood, such unusual behavior may be caused by inappropriate G-protein usage, overexpression of recombinant receptors, or other as yet unidentified phenomena. Our laboratory has noted that some chemokine receptors may not be expressed in a stable manner and that functional responses can be lost if not selected for repeatedly. Overexpression is a natural consequence of using a strong promoter and may lead to functional responses that are potentially deleterious to transfected cells. Some changes we have observed with GPCR transfectants are increases in cell adhesiveness or decrease in growth rate. With CCR11 our transfected cell population was initially selected by chemotaxis. When these cells were cloned, the majority had lost their responsiveness to MCP-4, but some clones responded even more vigorously than the original selected population. Thus, chemotactic selection greatly aided our identification and characterization of CCR11.

Compared with other characterized chemokine receptors, we observed only weak calcium mobilization in response to MCP-4 stimulation. Perhaps CCR11 signal transduction is linked to G-proteins that are not well complemented in L1.2 cells. Perhaps this receptor does not naturally induce a strong calcium response, like some other GPCRs. Alternatively, CCR11 calcium responses in L1.2 cells may be linked to cellular toxicity. Finally, CCR11 may recognize other, as yet unidentified, ligands that cause more significant calcium flux. Nevertheless, MCP-4...

\[ C. J. Raport and P. W. Gray, unpublished observations. \]
\[ V. L. Schweickart, B. Steiner, and P. W. Gray, unpublished observations. \]
is a major ligand for CCR11 as shown by its strong binding affinity and potent agonist activity in chemotaxis experiments.

As shown by Northern blot analysis, CCR11 has an unusual pattern of expression for a chemokine receptor. Because it is not highly expressed in lymphoid organs such as thymus or spleen, CCR11 is not likely to be involved in lymphocyte development as are CXCR4, CCR7, and CCR9 (Refs. 10–15). In addition, CCR11 is virtually undetectable in peripheral blood, being primarily expressed in the heart, small intestine, and lung. With the exception of CXCR4, which is broadly expressed in many tissues, chemokine receptors are typically expressed exclusively on cells of lymphoid or myeloid origin. Our inability to detect transcript in these cells may indicate that CCR11 is expressed on a subpopulation of lymphoid cells that are rare in whole blood but resident in specific tissues. CXCR3, for example, is expressed only on eosinophils and a subset of Th2 cells and is undetectable by Northern blot in peripheral blood (39, 40). Alternatively, CCR11 may be expressed on parenchymal cells and play a role currently unappreciated for chemokine receptors.

MCP-4 was determined. Panel B, displacement of the binding of $^{125}$I-MCP-4 to CCR11-transfected L1.2 cells with unlabeled MCP-4 (○), MCP-2 ( ●), MCP-3 (■), MCP-1 (●), and eotaxin (□). Cells were incubated with 0.1 nM $^{125}$I-MCP-4 in the presence of the indicated concentrations of unlabeled chemokine. Cells were washed three times in binding buffer, and the amount of bound $^{125}$I-MCP-4 was determined. Panel B, displacement of $^{125}$I-MCP-4 by other chemokines. L1.2 cells stably transfected with human CCR11 were incubated with 0.1 nM $^{125}$I-MCP-4 in the presence of the indicated concentrations of unlabeled chemokine. Cells were washed, and specific binding of $^{125}$I-MCP-4 was determined.
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CCR11 is a functional receptor for the monocyte chemoattractant protein family of chemokines.

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In this paper, we reported that CCR11 is a functional receptor for the MCP family of chemokines. Gosling and colleagues (Gosling, J., Dairaghi, D. J., Wang, Y., Hanley, M., Talbot, D., Miao, Z., and Schall, T. J. (2000) J. Immunol. 164, 2851–2856) simultaneously reported ELC, SLC, and TECK to be the ligands for this receptor (which they called CCR10). Upon further investigation, the transfected cell line used for our original study was found to express undetectable levels of CCR11 by Northern analysis. To generate this cell line, we utilized the selective method of chemotaxis to enrich for cells that responded to chemokines. The selected cells responded to MCP-4 and other members of the MCP family as reported, but new Northern analysis data show an up-regulation of the endogenous murine CCR2 in these cells. Furthermore, cells transfected with recombinant murine CCR2 have a ligand recognition pattern and chemotactic response similar to our reported CCR11. Consequently, we believe that our observed data were not due to CCR11 but instead may be attributable to up-regulation of the endogenous murine CCR2 gene. We have since expressed CCR11 in both L1.2 and HEK293 cells and confirmed high levels of expression with a CCR11-specific antiserum. Both cell lines demonstrate binding to ELC, SLC, and TECK. Therefore, we are in agreement with the results of Gosling et al. and have confirmed ELC, SLC, and TECK as ligands for CCR11. Other information in our report regarding the sequence, chromosomal localization, and tissue distribution of the receptor is accurate and not affected by this correction.

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