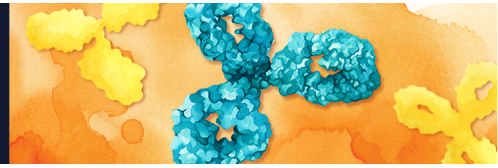


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J Immunol 1998; 160:1975-1981; ;
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



Identification of CCR8 as the Specific Receptor for the Human β -Chemokine I-309: Cloning and Molecular Characterization of Murine CCR8 as the Receptor for TCA-3¹

Iñigo Goya, Julio Gutiérrez, Rosa Varona, Leonor Kremer, Angel Zaballos, and Gabriel Márquez²

Chemokine receptor-like 1 (CKR-L1) was described recently as a putative seven-transmembrane human receptor with many of the structural features of chemokine receptors. To identify the ligand of CKR-L1, we have studied chemokine-induced calcium mobilization in 293 cells transfected with CKR-L1. Of 20 different chemokines tested, only I-309 was able to elicit a significant calcium mobilization. In addition, I-309 induced the transfectants to migrate in vitro. As expected for chemokine receptor-mediated effects, pertussis toxin, but not cholera toxin, inhibited both the calcium flux and migration of the CKR-L1 transfectants in response to I-309. All of these data support the conclusion that I-309 is a functional ligand for CKR-L1. According to the current chemokine receptor nomenclature, we have designated this gene as CCR8. The murine CCR8 (mCCR8) gene was cloned, and its predicted amino acid sequence showed a 71% identity with that of human CCR8. As human CCR8, mCCR8 is expressed in thymus. Both I-309 and its murine homologue TCA-3 were able to induce calcium mobilization in transiently transfected 293-EBNA cells expressing mCCR8. The affinity of the binding of ¹²⁵I-labeled TCA-3 to mCCR8 was high ($K_d \approx 2$ nM); the binding was prevented completely by an excess of cold TCA-3, and only partially competed (40%) by I-309. The identification of I-309 and TCA-3 as the functional ligands for CCR8 receptors will help to unravel the role of these proteins in physiologic and pathologic situations. *The Journal of Immunology*, 1998, 160: 1975–1981.

Chemokines are potent and specific chemoattractants for leukocytes, thus playing an important role in immunoregulatory processes, as well as in inflammatory and infectious diseases. They constitute a group of small related proteins that fall into two major subfamilies according to the presence of two typical cysteine residues near the N-terminal region: CXC or α -chemokines, in which X is any amino acid residue, and CC or β -chemokines (1–3). Recently, lymphotactin, a chemokine with only one cysteine residue in the N-terminal region (4), and fractalkine/neurotactin, a chemokine with a CX₃C motif (5, 6), have been described. α -chemokines exert their actions mainly on neutrophils, while β -chemokines stimulate a wide variety of cell types, such as monocytes, eosinophils, basophils, and lymphocytes.

Chemokine receptors are a family of single-chain, G protein-coupled proteins (7–10). Four receptors for α -chemokines have been reported. Two of them bind IL-8 (11, 12): CXCR1, which also binds GCP-2, and CXCR2, which also binds Gro- α , Gro- β , Gro- γ , NAP-2, and GCP-2 (13–15). CXCR3 binds IP-10 and monokine induced by IFN- γ (16), and SDF-1 is the only known

ligand for CXCR4 (17, 18). Concerning receptors for β -chemokines, seven have been described to date. The identified ligands for CCR1 are MIP-1 α ,³ RANTES, and MCP-3 (19–21). CCR2 is able to bind MCP-1, MCP-3, and MCP-4 (22–26). Eotaxin, RANTES, MCP-3, and MCP-4 are bound by CCR3 (26–29). CCR4 seems to bind only TARC (30), and CCR5 is a receptor for RANTES, MIP-1 α , and MIP-1 β (27). The most recently identified β -chemokine receptors are CCR6 and CCR7, which are able to signal upon the specific binding of LARC and ELC, respectively (31, 32).

Despite this degree of promiscuity, with most chemokine receptors being able to bind more than one chemokine, and many chemokines binding more than one receptor, some putative receptors still remain orphan (33–36) and the receptors for some chemokines are not known (4–6, 37). Unraveling the specificity of interactions between chemokines and their receptors would be of great help for understanding the molecular basis of the variety and specificity of chemokine activities.

We have reported recently the cloning of CKR-L1 and CKR-L3, two human genes that are expressed in some lymphoid tissues and are new putative chemokine receptors (38). The ligand for CKR-L3, the β -chemokine LARC, has been described very recently, and now this receptor is called CCR6 (31). However, no ligand for CKR-L1 has been reported to date. We now present evidence showing that CKR-L1 specifically binds the β -chemokine I-309. Therefore, according to the current chemokine receptor nomenclature, we designate CKR-L1 as CCR8. We also report the cloning of the mCCR8 receptor and data showing its specific binding of TCA-3, the I-309 mouse homologue.

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Received for publication August 18, 1997. Accepted for publication November 3, 1997.

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¹ This work was supported by Pharmacia & Upjohn (Barcelona, Spain). I.G. is the recipient of a Fellowship from Programa de Formación de Investigadores del Gobierno Vasco (Spain).

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³ Abbreviations used in this paper: MIP, macrophage-inflammatory protein; CRK, chemokine receptor-like; h, human; ¹²⁵I-TCA-3, ¹²⁵I-labeled TCA-3; m, murine; MCP, monocyte-chemotactic protein; ORF, open reading frame.

Materials and Methods

Chemokines and cell cultures

Recombinant human chemokines were obtained from PeproTech (London, UK) or R&D Systems (Minneapolis, MN). Recombinant mouse chemokines were bought from PharMingen (San Diego, CA) (TCA-3) or R&D Systems (C10). Human embryonic kidney 293 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD) and grown in DMEM supplemented with 10% FCS (Seralab, Crawley Down, U.K.) and antibiotics, at 37°C in a humidified 5% CO₂ atmosphere. The CCR8 genes were cloned in pCIneo (Promega Corp., Madison, WI), and the corresponding 293 stable transfectants were obtained by G418 selection of calcium phosphate-transfected cells, as described (39). 293-EBNA cells (Invitrogen Corp., San Diego, CA) were used as host for transient transfections. To study the effect of pertussis and cholera toxins on I-309-mediated calcium mobilization and migration of CCR8 transfectants, the cells were cultured in the presence of 0.1 µg/ml pertussis toxin or 0.4 µg/ml cholera toxin for 16 h before the assays. The mouse thymic lymphoma BW5147 cell line was obtained from ATCC and was grown in RPMI 1640 supplemented with 10% FCS (Seralab), 2 mM glutamine, 1 mM sodium pyruvate, and antibiotics.

Calcium mobilization assays

Variations in the intracellular concentration of calcium in stable transfected cells were analyzed by fluorimetry essentially as described by Didsbury et al. (40). Briefly, 293/CCR8-transfected cells were loaded with 3 to 5 µM Indo-1-AM (Molecular Probes, Leiden, The Netherlands) at a density of 10⁷ cells/ml in HBSS containing 20 mM HEPES (pH 7.3) and 0.1% BSA. After washing, the cells were resuspended in the same buffer at a density of 1 to 3 × 10⁶ cells/ml, and samples of 1.5 ml were placed in a cuvette in a heated holder equilibrated at 37°C, under stirring, in an SLM-8000C spectrofluorimeter. A wavelength of 350 nm was used for excitation; samples of chemokines in 15 µl were injected into the cuvette, while dual-fluorescence emission was detected continuously at 405 nm and over 500 nm. The ratio of fluorescence emission at each wavelength, F₄₀₅/F_{>500}, was recorded as a measure of calcium concentration. Calcium mobilization in transiently transfected cells was analyzed as described elsewhere (41). Briefly, plasmid pcytAEQ (Molecular Probes), encoding apoaequorin, was cotransfected into 293-EBNA cells along with each plasmid encoding the receptors to be tested. Twenty-four hours after transfection, the cells were loaded with hcp-coelenterazine to reconstitute the aequorin protein as a luminescent calcium probe. The loaded cells were analyzed for luminescence in the same way described for the fluorescence analysis, except that the samples were not excited and filters were not used for collecting the emitted light.

Chemotaxis assays

The migration of 293 cells stable transfected with hCCR8 was studied in a 48-well microchamber (Neuro Probe, Cabin John, MD) essentially as described elsewhere (21). Chemokines in different concentrations were loaded in the lower wells (27 µl/well), and cells (50 µl/well, 10⁶ cells/ml) in the upper wells. Polyvinylpyrrolidone-free filters with 10-µm pores (Poretics, Livermore, CA), precoated for 2 h at 37°C with type VI collagen (Sigma Chemical Co., St. Louis, MO), were used. The chamber was incubated for 5 to 6 h at 37°C in a humidified atmosphere with 5% CO₂. After incubation, filters were removed and the cells present in the upper part were wiped off. The filters were then fixed and stained with a crystal violet solution (0.5% crystal violet, 20% methanol). Blue spots developed at positions in which cell migration had occurred. Between 5,000 and 50,000 cells, the color intensity of the spots was checked to be directly proportional to the number of cells present in the filter. This allowed the quantification of the migration results by densitometry of the spots (National Institutes of Health Image software). A migration index was calculated by the following ratio: densitometric result of samples of cells migrated to chemokines/densitometric result of samples of cells migrated to buffer.

Cloning of mCCR8

A quantity amounting to 6 × 10⁵ plaque-forming units from a 129 SVJ mouse genomic DNA library in λGEM-12 vector was plated and screened with a ³²P-labeled DNA probe corresponding to the complete ORF of the hCCR8 gene. Positive plaques were rescreened until plaque-purified clones were obtained. Clone λ301 was shown to contain a complete ORF homologue to that of the hCCR8 gene. The identified mCCR8 sequence was subcloned into pCIneo to generate the corresponding expression vector.

Northern blot analysis

Total RNA from normal adult mouse tissues or the BW5147 murine T cell lymphoma was extracted using Tri-reagent (Sigma Chemical Co.), as recommended by the supplier. Poly(A)⁺ RNA was purified from the total RNA samples using oligo(dT) cellulose. Poly(A)⁺ RNA was fractionated by electrophoresis on a denaturing formaldehyde-agarose gel, transferred to a Nylon Hybond N⁺ membrane, and UV cross-linked. The membrane was prehybridized and then hybridized in Rapid Hyb buffer (Amersham, Little Chalfont, U.K.), as recommended by the supplier.

¹²⁵I-TCA-3 binding to 293/mCCR8 cells

Recombinant mouse TCA-3 (PharMingen) was iodinated as described previously (42). Briefly, 2 µg TCA-3 in 50 µl 0.1 M borate buffer, pH 8.5, was incubated in ice for 15 min with 1 mCi ¹²⁵I-labeled Bolton-Hunter reagent (Amersham). The reaction was stopped by adding 250 µl of 0.5 M glycine in the same buffer, and the nonincorporated ¹²⁵I was removed by chromatography in a Sephadex G-25 PD10 column (Pharmacia, Uppsala, Sweden). The sp. act. of radiolabeled TCA-3 was about 8 × 10⁸ cpm/µg. The binding assays using ¹²⁵I-TCA-3 were performed essentially as described (43). 293-EBNA cells were transfected transiently with an expression plasmid encoding mCCR8. After 48 h, 10⁶ cells were mixed with different concentrations of ¹²⁵I-TCA-3 in the presence or absence of a 100-fold molar excess of cold TCA-3, in a final volume of 300 µl of buffer A (RPMI 1640 containing 1% BSA and 20 mM HEPES, pH 7.4). After incubating the mixtures for 2 h at 4°C, cell suspensions were overlaid onto tubes containing 700 µl of cold FCS and they were spun. Cell pellets were washed with 900 µl of ice-cold buffer A and, finally, the radioactivity associated to cell pellets was counted in a gamma counter (Wallac Oy, Turku, Finland). In binding competition assays, 10⁶ cpm/sample of ¹²⁵I-TCA-3 was competed for 2 h at 4°C with a 170-fold molar excess of unlabeled TCA-3, or 250-fold molar excess of I-309, JE, mMCP-5, or C10, in 300 µl of buffer A.

Results

I-309-induced calcium mobilization in 293/hCCR8 cells

The interaction of I-309 with hCCR8 was studied by measuring changes in intracellular calcium concentration of 293 cell clones stable transfected with a hCCR8 expression plasmid (38) or with the void vector (pCIneo), upon addition of I-309 at different concentrations. Figure 1A depicts the results obtained with 293/hCCR8-transfected cells. The addition of 0.1 nM I-309 did not result in detectable calcium mobilization. Higher I-309 concentrations (0.3–1 nM) induced a significant wave of calcium mobilization, and peaks of increasing intensity were obtained upon stimulation with higher concentrations of the chemokine. As expected, a second addition of 100 nM I-309 to a sample previously stimulated with the same concentration of chemokine did not result in calcium mobilization. Other α- or β-chemokines such as IL-8, Gro-α, Gro-β, Gro-γ, ENA-78, IP-10, monokine induced by IFN-γ, NAP-2, PF-4, MCP-1, MCP-2, MCP-3, MCP-4, RANTES, MIP-1α, MIP-1β, HCC1, eotaxin, and lymphotactin did not elicit a significant calcium mobilization upon addition to hCCR8 transfectants (38, and results not shown). I-309 did not provoke calcium mobilization in the 293 control cells transfected with the void vector (Fig. 1B).

To study the possible role of G proteins in the signal-transduction pathway of hCCR8, 293/hCCR8 transfectants were incubated with pertussis or choleric toxins, as described in *Materials and Methods*. As shown in Figure 1C, calcium mobilization induced by I-309 was abolished completely by pretreatment of the cells with pertussis toxin, while the cells were still able to mobilize calcium in response to another receptor-mediated, pertussis-insensitive stimulus such as the addition of 50 µM ATP. Conversely, pretreatment of 293/hCCR8 cells with cholera toxin did not decrease their I-309-induced calcium response (not shown).

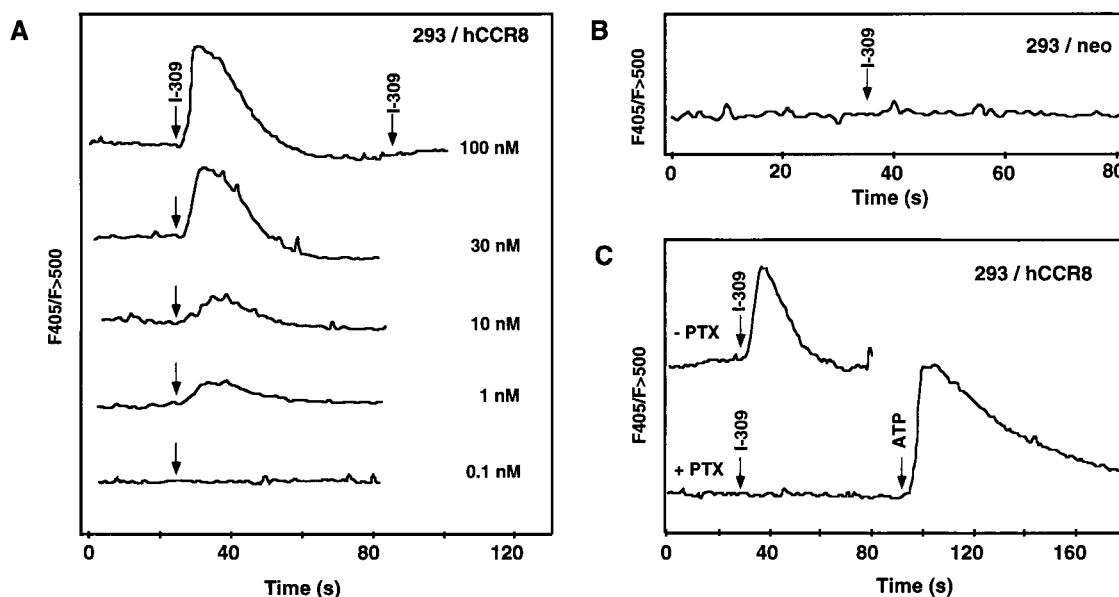


FIGURE 1. I-309-induced calcium mobilization in 293/hCCR8 stable transfectant cells. *A*, Indo-1-loaded hCCR8-transfected cells were stimulated with I-309 at the indicated concentrations. The 100 nM addition was repeated after 60 s. *B*, Indo-1-loaded control cells transfected with void vector were stimulated with 100 nM I-309. *C*, hCCR8-transfected cells were cultured in the absence or the presence of pertussis toxin, as indicated in *Materials and Methods*. The cells were then loaded with Indo-1 and stimulated with 30 nM I-309. ATP (50 μ M) was added 65 s after the chemokine in the case of toxin-treated cells. Arrows indicate the time of every addition. Alterations in the F405/F>500 ratio reflect changes in Ca^{2+} . The data shown in *A* and *B* are representative of three experiments; two independent clones of stable hCCR8-transfected cells yielded similar results. The data shown in *C* are representative of two experiments.

I-309-induced chemotaxis of 293/hCCR8 cells

Figure 2*A* shows that I-309 induced the specific migration of 293/hCCR8 cells. The I-309-dependent migration followed a dose-response curve typical of chemokines, with a maximum between 5 and 10 nM. Control 293 cells transfected with the vector alone did not significantly migrate to I-309. The effect of a pretreatment with toxins on the I-309-induced migration of 293/hCCR8 cells was also tested. As shown in Figure 2*B*, pertussis toxin practically abolished the migration induced by I-309, while cholera toxin did not significantly affect migration.

Cloning of mCCR8

By screening of a 129 SVJ mouse genomic library, we have isolated the murine homologue of hCCR8. A DNA probe consisting in the complete ORF of the hCCR8 gene was used to identify a positive clone, in which an ORF encoding a protein of 353 amino acid residues was identified. The comparison between this deduced amino acid sequence and that corresponding to the hCCR8 showed that the overall identity between both proteins was 71% (Fig. 3). In the extracellular N-terminal region, in which the murine protein is two amino acids shorter than the human one, the identity is 50%. The N8 amino acid residue of mCCR8 is a potential site for N-glycosylation. This is a very common structural feature of chemokine receptors, although it is not present in hCCR8. When the amino acid sequence of mCCR8 is compared with those of other murine β -chemokine receptors, the highest identity (43%) corresponds to mCCR4 (Fig. 3). Other mouse β -chemokine receptors share 40% (mCCR2 and mCCR5) and 37% (mCCR1 and mCCR3) identity with mCCR8 (Fig. 3). Given that the coding sequence of mCCR8 was obtained from genomic DNA and 5' ends of the coding sequences of several chemokine receptors have been shown recently to be interrupted by introns, we have used an anchored PCR-based strategy to analyze the 5' end of cDNA from the mCCR8 gene. Sequence analysis of PCR products did not detect

an alternative ATG codon (data not shown). Similarly, the hCCR8 genomic coding sequence is coincident with that of the cDNA (data not shown).

Murine CCR8 expression

The tissue distribution of mCCR8 was investigated by Northern blotting of poly(A)⁺ RNA obtained from different mouse organs. Using the complete mCCR8 ORF as a probe, two transcripts of about 2.2 and 1.5 kb were detected in thymus (Fig. 4). We also detected a strong expression of mCCR8 in the BW5147 mouse thymic lymphoma cell line (Fig. 4). The message was not detectable in the rest of tissues tested.

TCA-3-induced calcium mobilization in 293-EBNA/mCCR8 cells

Figure 5 shows the results obtained in calcium mobilization experiments when 293-EBNA transient transfectant cells expressing either the human or murine CCR8 receptor were stimulated with either I-309 or its murine homologue, TCA-3. Addition of 10 nM I-309 or 10 nM TCA-3 to the transfectants expressing mCCR8 provoked calcium mobilization, while addition of 100 nM C10, another murine chemokine, did not provoke any response. However, the cells expressing hCCR8 were only able to respond to I-309 and remained unresponsive upon addition of 100 nM TCA-3. As expected, control cells transfected with the void vector did not respond to the addition of I-309 or TCA-3.

mCCR8 binding of ¹²⁵I-TCA-3

Ligand-binding assays were performed with ¹²⁵I-TCA-3 on human 293-EBNA cells transiently transfected with an expression plasmid encoding mCCR8. The transfectants bound radiolabeled TCA-3 with high affinity (Fig. 6). Nonspecific binding, defined as that noninhibited by a 100-fold molar excess of unlabeled TCA-3, was approximately 25% of total binding. The specific binding data were plotted and showed the typical hyperbolic curve, which

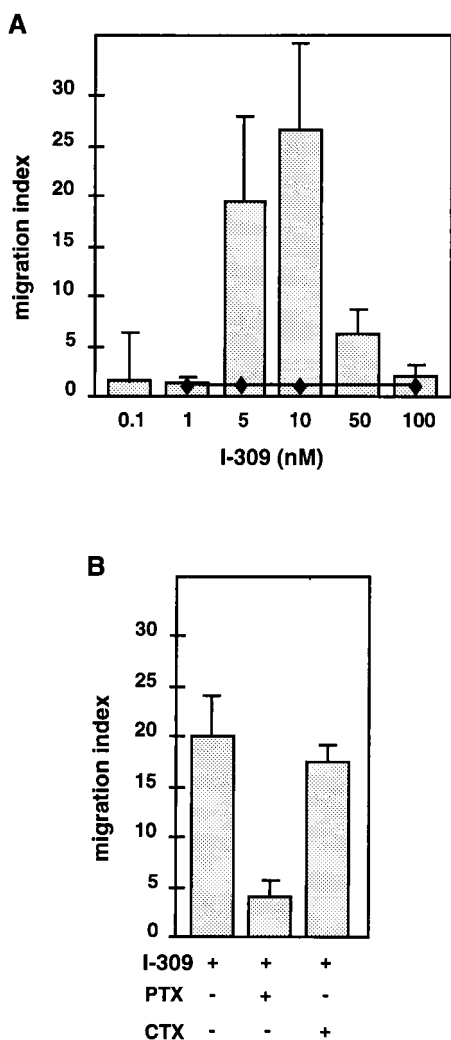


FIGURE 2. I-309-induced migration of 293/hCCR8 stable transfectant cells. **A**, In vitro migration experiments were conducted with 293 cells stable transfected with hCCR8 (bars) or the vector alone (♦). **B**, Stable 293/hCCR8-transfectant cells were treated with pertussis toxin (PTX) or cholera toxin (CTX), as indicated in *Materials and Methods*, and in vitro migration tests were then performed. I-309 (10 nM) was used as chemoattractant in these assays. Tests were done in triplicate or tetraplicate, and a migration index was estimated, as described in *Materials and Methods*. The data represent mean ± SE. Representative results from at least three different experiments are shown.

tended to a plateau at ¹²⁵I-TCA-3 concentrations over 2 nM (Fig. 6A). Scatchard analysis of these data was used to estimate an equilibrium dissociation constant (K_d) of 2 nM (Fig. 6B). ¹²⁵I-TCA-3 did not bind significantly to 293 cells transfected with the void vector (data not shown). Competitive binding inhibition assays using other chemokines were also conducted. Figure 6C shows that a 170-fold molar excess of unlabeled TCA-3 completely inhibited the binding of 10⁶ cpm of ¹²⁵I-TCA-3, which was competed only partially by a 250-fold molar excess of I-309. No inhibition of ¹²⁵I-TCA-3 binding was obtained with the same molar excess of other mouse chemokines tested, such as C10, JE, or mMCP-5.

Discussion

We have described recently a new human orphan putative chemokine receptor called CKR-L1 (38). The CKR-L1 gene is located in chromosome 3 and codes for a protein of 355 amino acid residues.

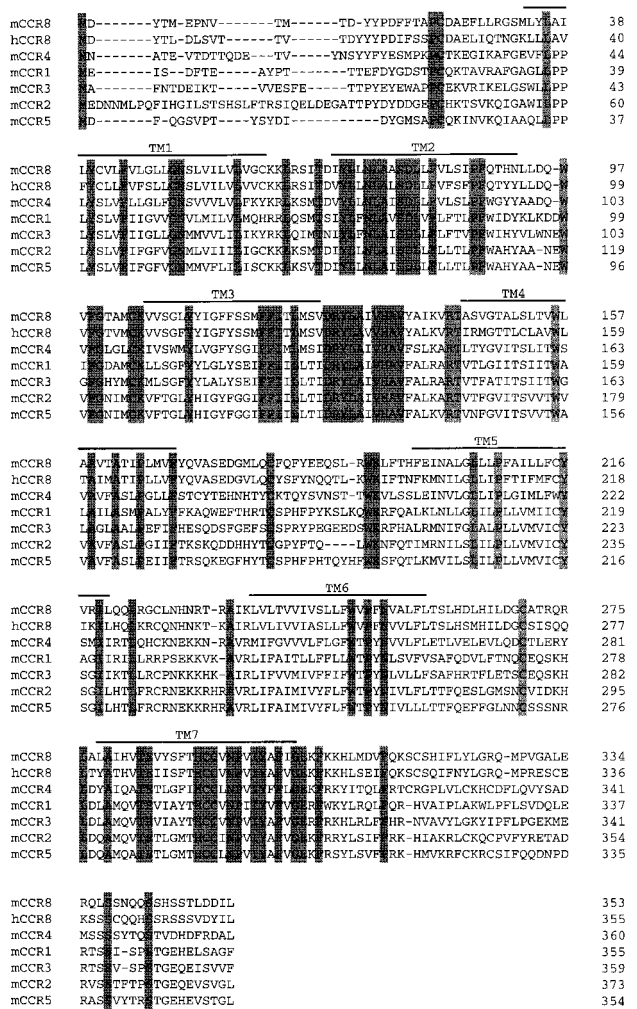


FIGURE 3. Alignment of the amino acid sequence of mCCR8 with those of hCCR8 and different murine β-chemokine receptors. Predicted transmembrane domains (TM) are marked. Identical residues are shaded in grey. N8 in mCCR8 sequence is a site for potential N-glycosylation. mCCR8 nucleotide sequence has been deposited in the EMBL database with accession number z98206.

Its predicted amino acid sequence is similar to that of β-chemokine receptors, sharing 42.3% of amino acid identity with CCR4. The analysis of CKR-L1 RNA expression by Northern blot showed that this receptor is expressed mainly in thymus and, to a lesser extent, in spleen. RTPCR studies on RNA samples from fractionated peripheral blood leukocytes showed that CKR-L1 is expressed in

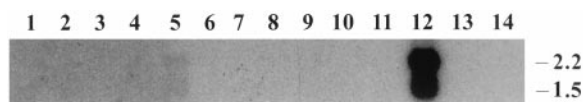


FIGURE 4. RNA expression analysis of mCCR8. Northern blot was performed with poly(A)⁺ RNA (2 μg/lane) prepared from normal adult mouse tissues or a mouse cell line, as described in *Materials and Methods*. The radiolabeled DNA fragment corresponding to the complete ORF of the mCCR8 gene was used as probe. Lanes: 1, lung; 2, smooth muscle; 3, colon; 4, small intestine; 5, thymus; 6, kidney; 7, testis; 8, spleen; 9, brain; 10, liver; 11, heart; 12, BW5147 T cell lymphoma; 13, bone marrow; and 14, lymph nodes. Transcript size in kilobases is shown on the right. The membrane was stained with methylene blue to estimate the amounts of RNA present in each lane (not shown).

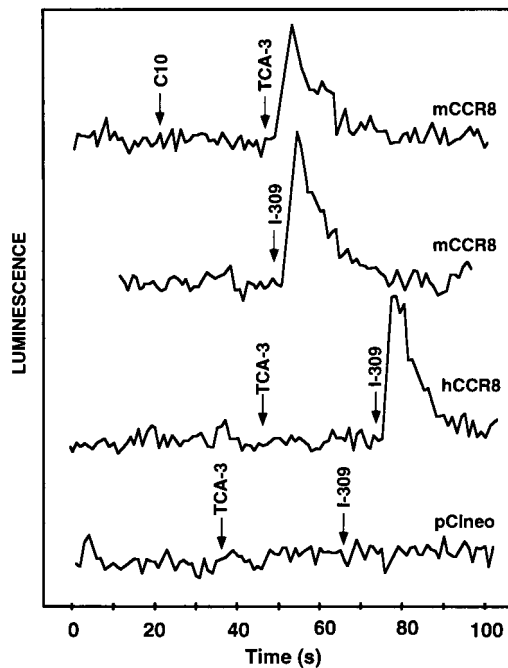


FIGURE 5. Chemokine-induced calcium mobilization in 293-EBNA cells transiently cotransfected with expression plasmids encoding apoaequorin and either mCCR8 or hCCR8. Samples of mCCR8-, hCCR8-, or mock-transfected (pCIneo) and hcp-coelenterazine-loaded cells were stimulated sequentially with different chemokines, as indicated. The chemokine concentrations used were 100 nM C10, 10 nM TCA-3, and 10 nM I-309, except for the hCCR8-transfected cells, which were stimulated with 33 nM TCA-3. Luminescence variations reflect changes in intracellular calcium concentration.

monocytes/macrophages, as well as CD4⁺ and CD8⁺ T lymphocytes. Similar results have also been reported by others (44, 45).

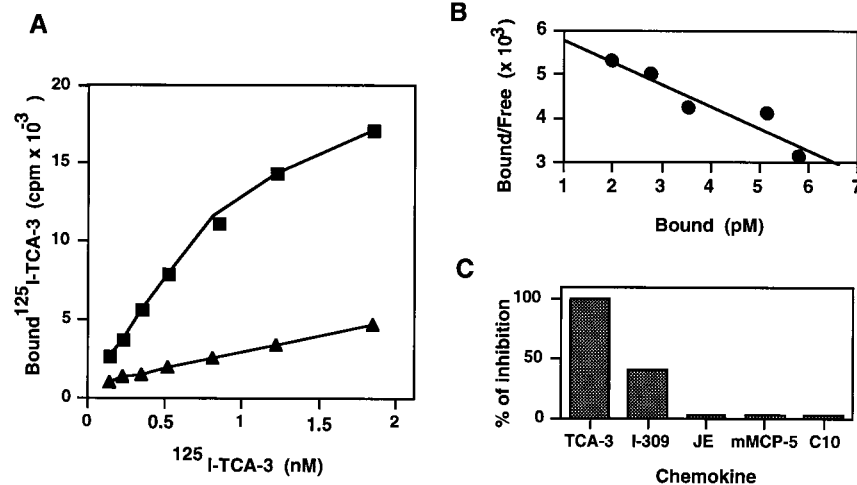
In the present study, we demonstrate that CKR-L1 is a specific receptor for I-309, a human β -chemokine that is produced by activated T lymphocytes (46). We therefore propose to refer to CKR-L1 as to hCCR8. At nanomolar concentrations, I-309 was able to mobilize intracellular calcium in 293 cells transfected with hCCR8. The stimulation of 293/hCCR8 cells with 100 nM I-309 resulted in the complete desensitization of the cells to a second similar stimulus. In addition, 293/hCCR8 cells were chemoattracted by nanomolar concentrations of I-309. Chemokine receptors are coupled to heterotrimeric G proteins, which are signal-

transduction mediators for this kind of receptors. Heterotrimeric G proteins constitute a family of effector proteins, some of which can be inhibited by pertussis or cholera toxins. As shown in Figures 1C and 2B, the I-309-induced mobilization of intracellular calcium and migration on hCCR8 transfectants were inhibited clearly by pertussis toxin, suggesting that hCCR8 is coupled to the G_{ei} class of G_α subunits (47). All of these data agree with the reported behavior of other chemokine receptors and support the conclusion that I-309 is a functional ligand for hCCR8. Indeed, while this manuscript was under revision, two other groups have also reported that I-309 is a specific ligand for this receptor (48, 49).

Since murine models are valuable tools to study the biologic functions of new genes, we decided to search for a putative mCCR8 receptor. A potential seven-transmembrane receptor 71% similar to hCCR8 was cloned, and the ligand-binding characteristics shown by this protein strongly indicate that this clone is indeed the mCCR8 receptor. Among murine β -chemokine receptors, mCCR8 shows the highest degree of amino acid homology to mCCR4. This is also the case when hCCR8 is compared with the other human chemokine receptors. Northern blot analysis of the RNA expression of mCCR8 showed that, like its human counterpart, this receptor is expressed mainly in thymus. In the mouse, two transcripts of 2.2 and 1.5 kb are detected, whereas a single CCR8 transcript of 4.3 kb is present in humans (38).

As expected for a murine homologue of hCCR8, TCA-3, the murine homologue of I-309, is able to induce calcium mobilization in transiently transfected 293-EBNA/mCCR8 cells. Moreover, ¹²⁵I-TCA-3 binds to these transient transfectants with a K_d of 2 nM. A similar K_d value has been reported for the binding of ¹²⁵I-TCA-3 to mouse monocyte/macrophage cell lines and mouse mesangial cells (43). I-309 was also able to induce a calcium mobilization response in transiently transfected 293-EBNA/mCCR8 cells. This was consistent with the binding competition results, which showed that I-309 was able to cause a 40% inhibition of the specific binding of ¹²⁵I-TCA-3 to mCCR8. In addition, these data agree with previous reports describing the activity of I-309 on murine cells (50). However, TCA-3 was not able to induce calcium mobilization on transient transfectant 293-EBNA cells expressing hCCR8 (Fig. 5), and the same result was obtained with stable transfected 293/hCCR8 cells (results not shown). Nevertheless, this somewhat more species-restricted ligand specificity of hCCR8 must be interpreted cautiously, since TCA-3 has been reported to stimulate calcium mobilization on human THP-1 cells (51). Whether this apparent inconsistency reflects differences in the quality of the protein preparations (insect cell-produced TCA-3

FIGURE 6. Binding of ¹²⁵I-TCA-3 to 293/mCCR8-transfectant cells. **A**, Total (■) and non-specific (▲) binding were obtained by incubating 293/mCCR8 cells with increasing amounts of ¹²⁵I-TCA-3, in the absence or presence of a 100-fold molar excess of cold TCA-3. **B**, Scatchard analysis of the specific binding data. **C**, Competition for the binding of 10⁶ cpm of ¹²⁵I-TCA-3 to mCCR8. A 170-fold molar excess of cold TCA-3 or a 250-fold molar excess of the rest of the indicated chemokines was used. In the competition experiments, assays were done in duplicate; the data represent the mean of one representative experiment from three independent experiments performed.



was used in our experiments; TCA-3 produced in CHO cells was used in Ref. 51), in the sensitivity of the techniques used to measure calcium mobilization, or the existence of a human TCA-3-sensitive receptor different to hCCR8 remains to be established. It must be noted, however, that Samson et al. (45) did not detect hCCR8 transcripts in THP-1 cells by Northern blot analysis, which suggests that, indeed, a different receptor might be involved.

I-309 has been reported to chemoattract human monocytes, but not neutrophils, in *in vitro* migration assays, and to increase cytoplasmic free calcium concentration in human peripheral blood monocytes, but not in lymphocytes or neutrophils (52). The sensitivity of monocytes to I-309 and the insensitivity of neutrophils are consistent with our previous observations of hCCR8 RNA expression in monocytes/macrophages, but not in granulocytes. However, the reasons for the lack of lymphocyte response are not clear, since hCCR8 RNA seems to also be expressed in peripheral blood CD4⁺ and CD8⁺ lymphocytes (38). As suggested, the apparent lack of a standard chemokine functionality of I-309 on lymphocytes might reflect a differential coupling of hCCR8 to signal-transduction molecules in different cell types (52). Actually, an effect of I-309 on lymphocytic cells has been reported: I-309 protects murine BW5147 T cell lymphoma against dexamethasone-induced apoptosis, an activity that is also shared by TCA-3 (50). Interestingly, this protection against apoptosis showed by I-309 is not exerted by other chemokines, and it is abolished completely in the presence of pertussis toxin (50), suggesting that CCR8 might be the receptor involved. As shown in Figure 4, Northern analysis of poly(A)⁺ RNA from BW5147 cells shows a strong expression of mCCR8 transcripts. Indeed, we have performed Scatchard analysis of the specific binding of ¹²⁵I-TCA-3 to BW5147 cells (data not shown), and obtained a *K_d* of 1.1 nM, close to that estimated for the TCA-3/mCCR8 interaction.

As reported before (51), the pattern of murine blood cells responsive to TCA-3 does not exactly match that of human cells responsive to I-309. In several assays, TCA-3 has been shown to stimulate neutrophils (53, 54), whereas, as indicated above, I-309 seems to be inactive on human neutrophils. This is interesting because β -chemokine activation of neutrophils is not usual, and now the availability of mCCR8 will help to determine the molecular basis for this difference between the human and mouse systems.

Unlike most chemokine receptors, which are able to bind several chemokines, hCCR8 only binds 1 of 20 tested. This fact indicates a remarkable specificity, although we presently do not exclude the possibility that new hCCR8 and mCCR8 ligands will appear as the number of members of the chemokine family keeps on growing. In any case, the identification of I-309 and TCA-3 as the functional ligands for CCR8 receptors will help to unravel the role these proteins play in different physiologic and pathologic situations. In this regard, we are generating CCR8 knockout mice as a valuable tool to study the *in vivo* functions of this receptor.

Acknowledgments

The comments and suggestions of Dr. C. Martínez-A. are greatly appreciated. We also thank Dr. F. Gavilanes for his help with the fluorimetry assays.

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