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Molecular cloning and functional expression of two monocyte chemoattractant protein 1 receptors reveals alternative splicing of the carboxyl-terminal tails

(chemokines/chemotaxis/seven-transmembrane-domain receptor)

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ABSTRACT **Monocyte chemoattractant protein 1 (MCP-1) is a member of the chemokine family of cytokines that mediate leukocyte chemotaxis. The potent and specific activation of monocytes by MCP-1 may mediate the monocytic infiltration of tissues in atherosclerosis and other inflammatory diseases. We have isolated cDNAs that encode two MCP-1-specific receptors with alternatively spliced carboxyl tails. Expression of the receptors in *Xenopus* oocytes conferred robust mobilization of intracellular calcium in response to nanomolar concentrations of MCP-1 but not to related chemokines. The MCP-1 receptors are most closely related to the receptor for the chemokines macrophage inflammatory protein 1α and RANTES (regulated on activation, normal T expressed and secreted). The identification of the MCP-1 receptor and cloning of two distinct isoforms provide powerful tools for understanding the specificity and signaling mechanisms of this important chemokine.**

Monocyte chemoattractant protein 1 (MCP-1) is a potent and specific monocyte agonist and chemoattractant (1, 2). It is produced by endothelial cells, smooth muscle cells, and macrophages in response to a variety of mediators, including platelet-derived growth factor (3), tumor necrosis factor α (4), lipopolysaccharide (5), and oxidized low density lipoproteins (6). MCP-1 has been implicated as an important factor in mediating monocytic infiltration of tissues in a wide variety of inflammatory diseases, including rheumatoid arthritis (7) and alveolitis (8), as well as in macrophage infiltration of tumors (9). In the latter case activation of monocyte/macrophages by MCP-1 may contribute to suppression of tumor growth in animal models (10). Monocyte invasion of the artery wall is a critical event in the initiation of atherosclerosis (11), and MCP-1 is highly expressed in the active, macrophage-rich areas of human atherosclerotic plaques (12, 13). The observation that oxidized low density lipoproteins stimulate MCP-1 production in vascular cells provides a possible link between atherogenic lipoproteins and the recruitment of monocytes to the artery wall (6).

Structurally, MCP-1 is a member of an emerging family of chemotactic cytokines known as the chemokines (or intercrines) (see refs. 14 and 15 for reviews). Chemokine family members are 8- to 10-kDa basic heparin-binding proteins that are related by both primary structure and the positions of four cysteines. The chemokine family has been divided into two subfamilies based on the arrangement of the first two cysteines. In the C-X-C subfamily the first two cysteines are separated by one amino acid; members of this branch include interleukin 8 (IL-8) and melanocyte growth stimulatory activity (MGSA), also known as GRO. In the C-C subfamily the first two cysteines are adjacent; members of the C-C branch include MCP-1, RANTES (regulated on activation, normal T expressed and secreted), and macrophage inflammatory proteins 1α and 1β (MIP-1α and MIP-1β). In general, C-X-C family members are potent neutrophil agonists, while C-C members are potent monocyte agonists.

Because of the probable importance of chemokines in a variety of inflammatory and disease processes, attention has recently been focused on the leukocyte receptors that mediate chemokine responses. Two closely related seven-transmembrane-domain receptors for the C-X-C chemokine IL-8 have been reported (16, 17). At least one of these two distinct gene products also signals in response to melanocyte growth stimulatory activity (18, 19). More recently, a receptor that bound and signaled in response to the C-C chemokines MIP-1α and RANTES was described (18, 20). These receptors do not account, however, for the robust and specific responses of monocytes to MCP-1, and cloning of the MCP-1 receptor has thus far been elusive. We now report the molecular cloning and functional expression of two receptors with alternatively spliced carboxyl tails that signal in response to nanomolar concentrations of MCP-1 in a highly specific manner.§

MATERIALS AND METHODS

Cells. MonoMac 6 cells (21) were obtained from the DSM German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and are derived from a human monocytic leukemia. THP-1, HL-60, and HEL cells were from the University of California Cell Culture Facility. All cells were grown in RPMI-1640 medium (GIBCO/BRL) supplemented with 10% fetal calf serum, 25 mM Hepes, and antibiotics. Cells were grown to log phase, loaded with the calcium probe Fura-2, and assayed by spectrofluorimetry (Hitachi, model F-2000) for changes in intracellular calcium in response to MCP-1 and other chemokines as described (22).

PCR of MonoMac 6 cDNA, Subcloning, and Sequencing of Novel Receptors. Total RNA was isolated from MonoMac 6 cells by the method of Chomczynski and Sacchi (23), and poly(A)$^+$ RNA was obtained by affinity chromatography on oligo(dT)-cellulose columns (Pharmacia). First-strand cDNA synthesis was performed, starting with 5 μg of MonoMac 6 poly(A)$^+$ RNA according to the supplier's instructions (Phar-

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Abbreviations: MCP-1, monocyte chemoattractant protein 1; RANTES, regulated on activation, normal T expressed and secreted; MIP, macrophage inflammatory protein; IL-8, interleukin 8; cRNA, complementary RNA.

§The cDNA sequences corresponding to the amino acid sequences reported in this paper have been deposited in the GenBank data base (accession nos. U03882 and U03905).

macia). Degenerate primers corresponding to conserved sequences in the second and third transmembrane domains of the MIP-1α/RANTES receptor and the IL-8 receptors were used in the PCR reaction with MonoMac 6 cDNA. The primers had *Eco*RI and *Xho* I restriction sites added to their 5′ ends. The PCR was carried out for 30 cycles, beginning with a 0.5-min incubation at 94°C, 2 min at 50°C, 1.5 min at 72°C, and a final elongation step at 72°C for 4 min. PCR products corresponding in size to those expected of a seven-transmembrane-domain receptor were subcloned in pBluescript SK(−) (Stratagene) and sequenced by using fluorescently labeled dideoxynucleotides (24).

Construction and Screening of a MonoMac 6 cDNA Library. A cDNA library was constructed in the vector pFROG, a modified version of pCDM6 that includes approximately 100 bases of 5′ untranslated *Xenopus* globin sequence just 3′ of the SP6 promoter, as described (25). First- and second-strand DNA synthesis was performed, starting with MonoMac 6 poly(A)$^+$ RNA. *Bst*XI linkers were added to the cDNA by ligation, and agarose gel electrophoresis was used to size-select for cDNA of 2 kb or greater. The ligated vector was electroporated into competent MC1061p3 cells. Analysis of the library revealed a total complexity of 22 million colony-forming units, with an average insert size of 2.3 kb. A total of 1,000,000 colonies were screened by hybridization with the novel PCR product under conditions of high stringency [50% (vol/vol) formamide/6× SSC/5× Denhardt's solution/0.1 mg of salmon sperm DNA per ml/0.1% SDS, at 42°C for 16 h]. One clone containing the A form of the receptor and two independent clones containing the B form were isolated. Sequences were analyzed and protein alignments were constructed by using GeneWorks software (IntelliGenetics) and a Macintosh computer.

Calcium Efflux Assay. The calcium efflux assay was performed essentially as described (25). The complementary RNA (cRNA) was prepared by SP6 RNA polymerase transcription from a *Not* I-linearized vector and was run on an agarose gel to confirm a single band of the expected size. *Xenopus laevis* oocytes were injected with 20 ng of cRNA in a total volume of 50 nl per oocyte 1 day after harvesting. After incubation in modified Barth's buffer for 2 days at 16°C, the oocytes were loaded with ^{45}Ca (50 μCi/ml, Amersham; 1 Ci = 37 GBq) for 3 h, washed for 1 h, and placed in groups of seven into wells of a 24-well dish in a volume of 0.5 ml. ^{45}Ca efflux was determined by collecting the medium at 10-min intervals and quantifying β emissions in a liquid scintillation counter. After a stable baseline had been achieved, agonists were added to the oocytes in Barth's medium for 10 min. Uninjected oocytes were used as controls. All cytokines were obtained from R & D Systems.

RESULTS

Since monocytes are difficult to isolate in quantity and they express fewer than 2000 high-affinity MCP-1-binding sites per cell (26), we first sought to identify a cultured cell line that responded well to MCP-1. MCP-1 induced intracellular calcium fluxes in the human monocytic leukemia-derived cell lines THP-1 and MonoMac 6, but not in undifferentiated human HL-60 cells or human erythroleukemia (HEL) cells (Fig. 1*A*). The response of the MonoMac 6 cells was dose dependent (Fig. 1*B*), with half-maximal stimulation at 4 nM MCP-1, in agreement with the reported K_d of 2 nM for binding to monocytes (26). The response of MonoMac 6 cells to MCP-1 could not be ablated by prior exposure to RANTES, whereas the response to RANTES was partially blocked by prior exposure of the cells to MCP-1 (Fig. 1*C*). These results are consistent with published data for THP-1 cells (27, 28) and suggest that MonoMac 6 and THP-1 cells express an MCP-1 receptor that is distinct from the MIP-1α/RANTES receptor. MonoMac 6 cells were chosen for further study because their response to MCP-1 was more consistent than that of the THP-1 cells.

To clone the MCP-1 receptor we adopted a PCR-based strategy using degenerate oligonucleotide primers corresponding to the conserved sequences NLAISDL in the second, and DRYLAIV in the third, transmembrane domains of the MIP-1α/RANTES receptor, the IL-8 receptors, and the "orphan receptor" known as HUMSTSR (GenBank accession no. M99293), which we had independently cloned and found not to confer MCP-1 responsiveness to transfected cells (I.F.C., S.J.M., and S.R.C.; unpublished results). Amplification of cDNA derived from MonoMac 6 cells with these primers yielded a number of PCR products corresponding in size to those expected for a seven-transmembrane-domain receptor. Analysis of the subcloned PCR products revealed cDNAs encoding the predicted fragments of the receptors from which the primers were designed as well as one cDNA which appeared to encode a protein related to the MIP-1α/RANTES receptor. To obtain a full-length version of this clone a MonoMac 6 cDNA library was constructed in pFROG, a modified version of pCDM6, and probed by hybridization with

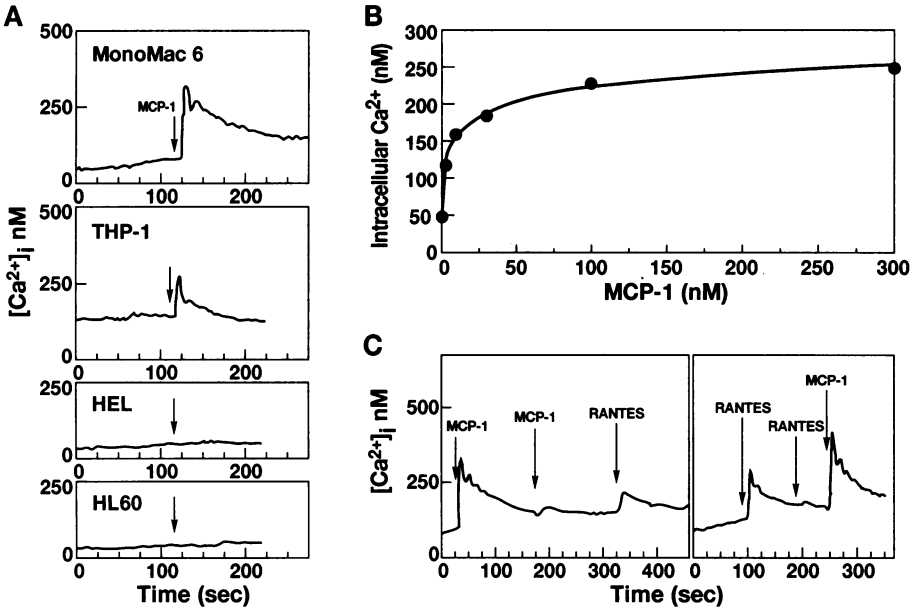

FIG. 1. MCP-1-induced calcium fluxes in human monocytic cell lines. Cells were grown to log phase, loaded with the calcium probe Fura-2, and assayed by spectrofluorimetry for changes in intracellular calcium concentration ($[Ca^{2+}]_i$) in response to agonists. (*A*) The human monocytic leukemia cell lines MonoMac 6 and THP-1, but not undifferentiated HL-60 cells or human erythroleukemia (HEL) cells, were activated by MCP-1 (100 nM; arrows). (*B*) Dose–response of MonoMac 6 cells to MCP-1. The EC_{50} is 4 nM. (*C*) Homologous and heterologous desensitization of MonoMac 6 cells to MCP-1 and RANTES (100 nM).

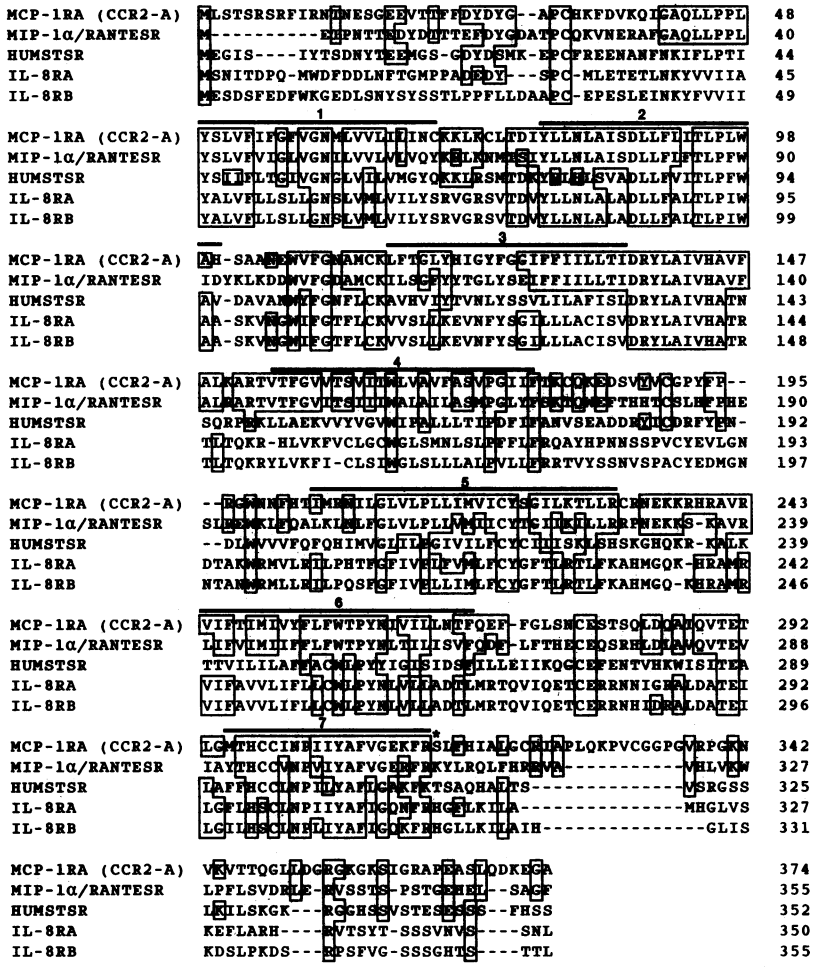

FIG. 2. Predicted amino acid sequence of the MCP-1 receptor. Shown is an alignment of the MCP-1 receptor clone CCR2-A with the MIP-1α/RANTES receptor (18, 20), the orphan receptor HUMSTSR, and the two IL-8 receptors (16, 17). Identical residues are boxed. The seven putative transmembrane domains are indicated by the horizontal bars. Gaps that have been inserted to optimize the alignments are indicated by dashes. The asterisk indicates the putative splice junction of the carboxyl-terminal tail. The MIP-1α/RANTES receptor is 51% identical to CCR2 at the amino acid level; the IL-8 receptors are 30% identical; and the orphan receptor known as HUMSTR is 31% identical.

this PCR product. A cDNA clone of 2.1 kb was obtained and termed CCR2 to denote the second C-C chemokine receptor to be identified. CCR2 contained a probable initiation methionine (29) followed by an open reading frame encoding a 374-amino acid protein [Fig. 2, MCP-1RA (CCR2-A)]. Hydropathy analysis of the predicted amino acid sequence of CCR2-A revealed seven putative transmembrane domains and, by analogy with the IL-8 and MIP-1α/RANTES receptors, an extracellular amino terminus of 48 residues.

Analysis of additional clones in our MonoMac 6 cDNA library revealed a second sequence that was identical to CCR2 from the 5′ untranslated region through the putative seventh transmembrane domain and then was completely different in the cytoplasmic tail and 3′ untranslated region. Two independent clones in our unamplified library were found to contain this sequence, which appears to represent alternative splicing of the carboxyl-terminal tail of the CCR2 protein. Alignment of these sequences with other chemokine receptors revealed that one of the CCR2 clones (CCR2-B) had a carboxyl tail that was 36% identical to the corresponding region in the MIP-1α/RANTES receptor (Fig. 3). The carboxyl tail of the other CCR2 clone (CCR2-A) bore no significant identity with other known proteins.

Given the relatedness of MCP-1 to the C-C chemokines MIP-1α and RANTES and the significant sequence identity between the CCR2 clones and the MIP-1α/RANTES receptor, we considered CCR2-A and -B to be strong candidates for the MCP-1 receptor. To determine whether one or both of these clones conferred responsiveness to MCP-1 or other chemokines, we microinjected cRNA from each clone into *Xenopus* oocytes and assayed signaling by measuring agonist-induced calcium mobilization. Signaling by the MIP-1α/RANTES receptor (cloned from the same MonoMac 6 cDNA library) and the IL-8 receptor (a generous gift of W. Wood, Genetech) was examined in parallel. Both of the CCR2 clones conferred robust and remarkably specific responses to nanomolar concentrations of MCP-1. The maximal response of the CCR2-B clone was marginally higher than that of

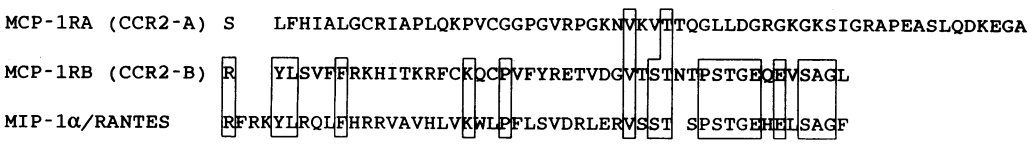

FIG. 3. Alignment of the carboxyl-terminal tails of the two MCP-1 receptor isoforms (CCR2-A, CCR2-B) and the MIP-1α/RANTES receptor.

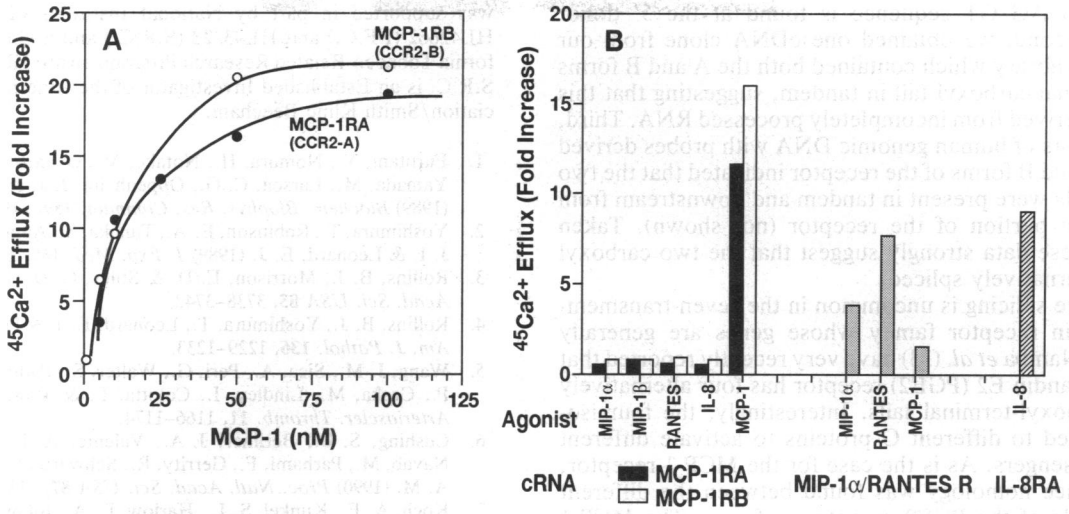

FIG. 4. Expression of the two MCP-1 receptor clones (CCR2-A, CCR2-B) and the MIP-1α/RANTES receptor in *Xenopus* oocytes. Oocytes were injected with 20 ng of cRNA encoding the MCP-1 receptors, the MIP-1α/RANTES receptor, or the IL-8A receptor. Two days after injection the oocytes were challenged with the indicated chemokines and agonist-induced $^{45}Ca^{2+}$ efflux was measured. (*A*) Dose–response curve for MCP-1. The EC_{50} is 10–15 nM. (*B*) Ligand specificity of the MCP-1 receptor. All chemokines were used at a final concentration of 500 nM except MCP-1, which was 250 nM. Oocytes injected with IL-8 receptor A cRNA were used as a positive control. The data are the average of three replicate determinations of each point and are representative of three separate experiments. No increase in $^{45}Ca^{2+}$ efflux was observed in uninjected oocytes (not shown).

CCR2-A, but the EC_{50} for MCP-1-induced $^{45}Ca^{2+}$ release was comparable for both (10–15 nM, Fig. 4*A*). No response was elicited by the related chemokines MIP-1α, MIP-1β, RANTES, or IL-8, even when these ligands were present at 500 nM (Fig. 4*B*). In contrast, the MIP-1α/RANTES receptor signaled in response to MIP-1α and RANTES but not MCP-1, consistent with published results (18, 20).

Northern blots of hematopoietic cell lines probed for each of the CCR2 clones revealed that both mRNA species migrated as a single 3.5-kb band (Fig. 5). Further, both mRNAs were expressed at approximately equal levels in the MCP-1-responsive cells (THP-1 and MonoMac 6) but neither was expressed in the unresponsive HEL or HL-60 cells. Each of the mRNAs was also expressed in freshly isolated human monocytes as demonstrated by reverse transcription PCR (not shown).

DISCUSSION

Previous pharmacological studies using THP-1 cells and monocytes have provided evidence for two receptors for MCP-1 (27, 28). One receptor displayed broad specificity and bound RANTES with high affinity and MIP-1α and MCP-1 with lower affinities. The recently cloned MIP-1α/RANTES receptor fits these criteria (18, 20). The second receptor appeared to be a high-affinity receptor specific for MCP-1, which has heretofore been elusive. In this paper we report the cloning of two receptors with alternatively spliced carboxyl-terminal tails that are specific high-affinity receptors for MCP-1. These receptors are closely related to the MIP-1α/RANTES receptor, transduce signals in response to nanomolar concentrations of MCP-1 in a highly specific manner, and appear to be the high-affinity MCP-1-specific receptor predicted by the pharmacological studies. Northern blot analysis of MCP-1-responsive and -nonresponsive cell lines was consistent with this interpretation. From this point forward we refer to the two CCR2 clones as the "A" and "B" forms of the MCP-1 receptor.

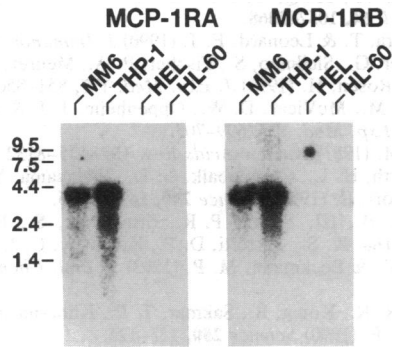

FIG. 5. Northern blot analysis of MCP-1-responsive [MonoMac 6 (MM6) and THP-1] and nonresponsive (HEL and HL60) cells with MCP-1RA (CCR2-A) and MCP-1RB (CCR2-B) cDNA probes. Poly(A)$^+$ RNA (7.5 μg) from each of the indicated cell lines was run on a 0.8% agarose/formaldehyde gel. The blots were probed by hybridization under conditions of high stringency with cDNAs from the carboxyl-terminal tail of the A and B forms of the MCP-1 receptor. Scale on left is kb.

Analysis of the amino acid sequence of the MCP-1 receptors revealed several interesting features. First, the MCP-1A receptor is 51% identical to the MIP-1α/RANTES receptor but has diverged significantly at its amino-terminal domain. The amino termini of the receptors for thrombin (25), thyrotropin (30), and IL-8 (31) all participate in binding their respective ligands, suggesting that divergence of the amino-terminal domains between the MCP-1 and MIP-1α/RANTES receptors may contribute to their different agonist specificities. The relatedness of these two receptors and their C-C chemokine ligands suggests that receptor chimeras may be used to test this hypothesis. Second, a striking identity between the MCP-1 receptor and the MIP-1α/RANTES receptor is found in the sequence IFFIILLTIDRY-LAIVHAVFAL(K/R)ARTVTFGV, which occurs at the end of the third transmembrane domain (Fig. 2). The corresponding region of rhodopsin is known to participate in G-protein binding (32), suggesting that this domain may mediate aspects of G-protein activation common to receptors for C-C chemokines.

Several lines of evidence support the hypothesis that the A and B forms of the MCP-1 receptor represent alternatively spliced variants of a single gene. First, the nucleotide sequence of the cDNAs of the A and B forms, including the 5′ untranslated sequence, is identical up to the putative splice

site, and an AG/GT sequence is found at the 5′ donor junction. Second, we obtained one cDNA clone from our MonoMac 6 library which contained both the A and B forms of the terminal carboxyl tail in tandem, suggesting that this clone was derived from incompletely processed RNA. Third, Southern blots of human genomic DNA with probes derived from the A and B forms of the receptor indicated that the two carboxyl tails were present in tandem and downstream from the common portion of the receptor (not shown). Taken together, these data strongly suggest that the two carboxyl tails are alternatively spliced.

Alternative splicing is uncommon in the seven-transmembrane-domain receptor family whose genes are generally intronless. Namba *et al.* (33) have very recently reported that the prostaglandin E2 (PGE2) receptor has four alternatively spliced carboxyl-terminal tails. Interestingly, the four isoforms coupled to different G proteins to activate different second messengers. As is the case for the MCP-1 receptor, little sequence homology was found between the different carboxyl tails of the PGE2 receptor isoforms. The MCP-1 receptor is the only other known example of alternative splicing of the carboxyl tails of seven-transmembrane-domain receptors. The closely related MIP-1α/RANTES and IL-8 receptors are believed to be intronless (16, 17, 20). Although both forms of the MCP-1 receptor elicited calcium mobilization in *Xenopus* oocytes it is not yet known if they activate the same second messengers in mammalian cells. By analogy with the PGE2 receptor, alternative splicing of the MCP-1 receptor carboxyl tails may represent a mechanism to increase the diversity of cellular responses to this important chemokine.

Monocytes and MonoMac 6 cells undergo homologous desensitization in response to multiple challenges with MCP-1 (ref. 18 and Fig. 1), and similar results have been reported for IL-8, MIP-1α, and RANTES (18, 34). The mechanism of homologous desensitization to chemokines is not well understood, but this phenomenon is likely to play an important role in limiting leukocyte responses *in vivo*. One commonly used mechanism for inactivation of seven-transmembrane-domain receptors is phosphorylation of the serines and/or threonines in the carboxyl-terminal tails by the β-adrenergic receptor kinase, or "BARK" enzyme (see ref. 35 for a review). It is of interest in this regard that the A form of the MCP-1 receptor has a total of 5 serines and threonines, while the B form has 10. Differences in receptor phosphorylation by a BARK-like enzyme might lead to differences in the kinetics of deactivation between the two forms of the MCP-1 receptor, providing another mechanism for increasing the diversity of cellular responses to MCP-1.

In summary, we report the cloning, functional expression, and signaling characteristics of two specific high-affinity receptors for MCP-1 with alternatively spliced carboxyl-terminal tails. These receptor cDNAs provide powerful tools for defining the molecular basis of chemokine–receptor interactions, as well as for understanding the role of MCP-1 in regulating monocyte/macrophage infiltration in a variety of disease processes. The MCP-1 receptors represent potentially important targets for therapeutic intervention in diseases in which monocyte/macrophage infiltration plays a significant role. The ability to define the expression pattern and binding characteristics of individual chemokine receptors will greatly aid our efforts to understand their roles in specific disease processes and ultimately in the development of appropriate pharmaceuticals.

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