The Amino-terminal Extracellular Domain of the MCP-1 Receptor, but Not the RANTES/MIP-1α Receptor, Confers Chemokine Selectivity

EVIDENCE FOR A TWO-STEP MECHANISM FOR MCP-1 RECEPTOR ACTIVATION*

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The chemotactant cytokines, MCP-1 (monocyte chemoattractant protein) and MIP-1α (macrophage inflammatory protein), are recognized by highly homologous but distinct receptors. To identify receptor domains involved in determining ligand specificity, we created a series of chimeric MCP-1 and RANTES (regulated on activation, normal T cell expressed and secreted) receptors that progressively interchanged the amino terminus and each of the three extracellular loops. Radiolabeled MCP-1 bound with high affinity to the wild-type MCP-1 receptor, but not to the RANTES/MIP-1α receptor (C-C CKR-1). Chimeras that retained the amino-terminal extension of the MCP-1 receptor bound MCP-1 with high affinity. In contrast, chimeric MCP-1 receptors, in which the wild-type amino terminus was replaced with the corresponding portion of the RANTES/MIP-1α receptor, bound MCP-1 with low affinity. These data indicate that the amino terminus of the MCP-1 receptor is necessary for high affinity binding of the ligand. Very different results were obtained using the RANTES/MIP-1α receptor. Radiolabeled MIP-1α bound with high affinity to chimeras that expressed the extracellular loops of the RANTES/MIP-1α receptor. In contrast to the MCP-1 receptor, substitution of the wild-type amino-terminal extension had little or no effect on MIP-1α binding. For the MCP-1, but not the RANTES/MIP-1α receptor, the presence of the wild-type amino terminus also significantly lowered the ligand concentration required for maximal signaling. We conclude that the amino-terminal extension of the MCP-1 receptor, but not the RANTES/MIP-1α receptor, is critically involved in ligand binding and signal transduction. These data reveal significant functional differences between the two C-C chemokine receptors and suggest a two-step mechanism for activation of the MCP-1 receptor.

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† The abbreviations used are: C5a, fifth component of complement; MCP-1, monocyte chemoattractant protein 1; RANTES, regulated on activation, normal T cell expressed and secreted; MIP-1α, macrophage inflammatory protein 1α; IL, interleukin; MCP-3, MCP-1 receptor; nt, nucleotide; RANTES/MIP-1αR, RANTES/MIP-1α receptor; PCR, polymerase chain reaction.
tor (25), as well as medium-sized peptides (e.g., neuropeptide Y (26)), important interactions with the extracellular loops have been identified. The mechanism of receptor activation by the chemokines is not well understood, but studies of the binding of IL-8 (27–29) and C5a (30, 31) have implicated the amino-terminal extension and one or more extracellular loops as directly interacting with the ligands.

We have investigated the sites of chemokine binding and activation of the MCP-1 and the RANTES/MIP-1α receptors. Taking advantage of the high degree of amino acid sequence conservation between these two receptors, we have created a series of chimeras in which the amino-terminal extension, and each of the three extracellular loops (including the transmembrane domains) of the RANTES/MIP-1α receptor was progressively substituted for the corresponding region of the MCP-1 receptor, and vice versa. In this paper, we report that the amino terminus of the MCP-1 receptor is necessary for high affinity binding of 125I-labeled MCP-1, but that other regions of the receptor are required to mediate signal transduction. In contrast, binding and signal transduction induced by MIP-1α do not appear to require the amino terminus, but do require the presence of the third extracellular loop of the RANTES/MIP-1α receptor. These data suggest a two-step model in which high affinity binding of MCP-1 by the receptor amino terminus allows subsequent low affinity interactions with the extracellular loops/transmembrane domain bundles to effect receptor activation and signaling.

MATERIALS AND METHODS

Construction of Chimeric Receptors—Receptor chimeras were constructed from cDNAs of MCP-1R (Type B; Ref. 11) and the RANTES/MIP-1α receptor (12), and nucleotide (nt) numbers correspond to GenBank accession numbers U03905 and L10918, respectively. We chose to study the Type B, rather than the Type A receptor, because the latter is poorly expressed in HEK-293 cells. Each receptor contains the proline signal sequence, followed by the Flag epitope sequence (32) fused to the Flag sequence at a conserved site in RRRR and a partial digest in MMMM each (Invitrogen, San Diego, CA) to yield the tagged wild-type receptors. The designs MRRR, mammalian expression of receptors in mammalian cells, were sequenced at the junctions used to create them by subcloning, and chimera 2 were sequenced immediately upstream of the second translation codon (nt 84, MCP-1R; and nt 66, C-C CKR-1). The proline signal/Flag sequence was subcloned into pBluescript and the receptor cDNA fused to the Flag sequence was subcloned into pcDNA3 (Invitrogen, San Diego, CA) and the tagged wild-type receptors. The designs MRRR and RANTES/MIP-1αR constructs containing the leader sequence was then subcloned into the corresponding sites of the expression vector pcDNA3 (Invitrogen, San Diego, CA) to yield the tagged wild-type receptors. The designs MRRR, mammalian expression of receptors in mammalian cells, were sequenced at the junctions used to create them by subcloning, and chimeras 2–3 were sequenced throughout the entire coding region to rule out mutations due to PCR amplification.

Chimeras 1 and 2—Receptors with the amino-terminal extension exchange were created by using a conserved Apal site in RRRR and MMMM. An Apal digest in MMMM and a partial digest in RRRR each yielded approximately a 1.8-kb fragment that was then subcloned into the Apal site in RRRR and MMMM, respectively, to yield RMMR and MRRR. Thus, in RMMR amino acids 1–32 are from the RANTES/MIP-1αR, while in MRRR amino acids 1–40 are from the MCP-1R. Amino acid numbers are from Ref. 11.

Chimeras 3 and 4—Receptors that exchange the amino terminus and first extracellular loop were created by “overlapping PCR” (33). The complementary primers A (5′-agataccgtctgtaagcgaccgc-3′) and B (5′-ggctggacagagcggctgg-3′) correspond to the conserved sequence RY-LAIVHA, nt 492–514 and 453–476 of MCP-1RB and RANTES/MIP-1αR, respectively. The amino-terminal and carboxyl-terminal halves of MRRR and RRRR were amplified in one round of PCR. A second round of PCR was performed using vector-specific primers that flank the cDNA insert, and purified fragments of the amino-terminal half of MRRR and the carboxyl-terminal half of RRRR as templates to create MRRR. To generate chimera RMMR, a second round of PCR was performed using purified fragments of the amino-terminal half of RRRR and the carboxyl-terminal half of MRRR as templates. The amplified products were purified and digested with HindII and NotI and cloned into the respective sites in pcDNA3. Thus in MRRR, amino acids 1–127 are from the MCP-1 receptor, and amino acids 1–120 in RRRM are from the RANTES/MIP-1αR.

Chimeras 5 and 6—Receptors that exchange the amino terminus and first and second extracellular loops were also created by overlapping PCR. The complementary primers C (5′-ttatgctgattgctgctg-3′) and D (5′-gtagctgacagctgcagtc-3′) correspond to the sequence MVICY, nt 730–746 and 703–719 of MCP-1RB and RANTES/MIP-1αR, respectively. The amino-terminal and carboxyl-terminal halves of MRRR and RRRR were amplified in one round of PCR. A second round of PCR, using primers specific primers that flank the D site in RRRR and purified fragments of the amino-terminal half of MRRR and carboxyl-terminal half of RRRR as templates, was performed to generate MRRM. RRRM was constructed in the same manner. Thus in MMRM, residues 1–209 are from the MCP-1R, and in RRRM, amino acids 1–206 are from the RANTES/MIP-1αR. The amplified products were purified and digested with HindII and NotI and cloned into the respective sites in pcDNA3. Thus in MMRM, amino acids 1–127 are from the MCP-1 receptor, and amino acids 1–120 in RRRM are from the RANTES/MIP-1αR.

Transfection and Fluorescence-activated Cell Sorting Analysis—HEK-293 cells (1573-CRL, American Type Culture Collection, Rockville, MD) were cultured in minimal essential medium with Earle’s balanced salt solution supplemented with 10% fetal bovine serum, and 100 μg/ml streptomycin. Cells were transfected with Lipofectamine (Life Technologies, Inc.) according to the manufacturer’s suggested protocol and placed under antibiotic selection, 800 μg/ml G418 (Life Technologies, Inc.). Cells of G418-resistant cells were analyzed for cell surface expression of receptors by a fluorescence-activated cell sorter. Approximately 1 × 10⁹ harvested cells were incubated at room temperature for 2 h with a culture medium containing a Flag epitope-specific antibody (M1; IB, New Haven, CT) diluted 1:1000. Cells were washed three times with phosphate-buffered saline and resuspended in culture medium containing goat anti-mouse IgG-FITC (Zymed Laboratories, South San Francisco, CA) diluted 1:1000 and incubated at room temperature for 30 min. Unbound antibody was removed by washing with phosphate-buffered saline, and the cells were resuspended in phosphate-buffered saline plus 10% fetal bovine serum and 0.5% bovine serum albumin (1:1000 dilution) and incubated in the dark at 27°C in an A3TA Biotoptics shaking set 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. Unbound ligand was removed by washing with 4 ml of buffer (10 mM Heps, 0.5 mM NaCl) over a 100-fold excess of unlabeled ligand, to 0.5 × 10⁶ cells in polypropylene tubes in a total volume of 300 μl (50 mM Heps, pH 7.4, 10 mM CaCl₂, 5 mM MgCl₂, 0.5% bovine serum albumin) and incubating the cells at 27°C in an A3TA Biotoptics shaking set shaking at 150 rpm. The samples were collected, washed, and counted as above. The data were analyzed using the curve-fitting programs Prism (GraphPad Inc., San Diego, CA) and the iterative nonlinear regression program LIGAND (34).

Adenylyl Cyclase Assays—Inhibition adenylyl cyclase was assayed as described (35). Briefly, stably transfected HEK-93 cells were grown until confluent in 24-well tissue-culture dishes and labeled overnight with 2 μCi/ml [methyl-3H]adenosine (25–30 Ci/mmol) in minimal essential medium–Earle’s balanced salt solution supplemented with 10% fetal calf serum. The cells were stimulated by addition of fresh medium containing either chemokine alone, forskolin alone (10 μM), or chemokine plus forskolin, all in the presence of 1 mM 3-isobutyl-1-methylxanthine, for 20 min at room temperature. The cAMP pool for each sample was normalized to its own ATP pool and

L.-M. Wong and I. F. Charo, unpublished data.
expressed as a ratio by the equation (cAMP cpm/ATP cpm) × 100. In each experiment, full dose-response curves were generated and expressed as a percent of the maximal stimulation achieved by forskolin alone. All data points were determined in duplicate.

RESULTS

Receptor Chimeras—To identify potential ligand-binding and signaling domains of the MCP-1 receptor, we utilized its high degree of relatedness to the RANTES/MIP-1α receptor. These two C-C chemokine receptors are 51% identical at the amino acid level but, with the exception of MCP-3 (36, 37), have little overlap in ligand specificity. MCP-1RB and the RANTES/MIP-1α-R are most highly related in their transmembrane domains (greater than 70% identical), but diverge considerably in their extracellular domains. We therefore generated a series of chimeric receptors in which the amino-terminal extensions and each of the three extracellular loops were progressively exchanged between the two receptors (Fig. 1). The wild-type MCP-1 receptor (Type B) is designated MMMM, to denote the amino terminus and each of its three extracellular loops. Similarly, the wild-type RANTES/MIP-1α receptor is designated RRRR.

Expression of Receptor Chimeras in HEK-293 Cells—HEK-293 cells were stably transfected with the wild-type and chimeric receptors. The Flag epitope (DYKDDDD) (32) was added at the amino terminus of each receptor to allow determination of receptor expression. The addition of this 8-amino acid sequence did not alter the binding or signaling properties of the MCP-1 or RANTES/MIP-1α receptors (data not shown). Surface expression was assayed by flow cytometry. Cell lines in which the chimeras were expressed at the cell surface were selected for further study (Fig. 2). Two chimeras in which the amino terminus was completely removed (ΔMMM and ΔRRR) were not detected at the cell surface (data not shown).

Binding of 125I-labeled MCP-1 and MIP-1α to Constructs with Exchanged Amino-terminal Extensions—We first examined the specific binding of 125I-labeled MCP-1 and MIP-1α to constructs in which the amino-terminal extensions of the wild-type receptors were exchanged. Labeled MCP-1 bound well to the wild-type MCP-1 receptor (MMMM), as well as to the chimera MRRR, which contained only the amino terminus of the MCP-1 receptor (Fig. 3A). Analysis of these data by the method of Scatchard revealed essentially identical equilibrium dissociation constants (Kd) of 0.33 nM for MMMM and 0.27 nM for MRRR (Fig. 3B). In contrast, the wild-type RANTES/MIP-1α receptor (RRRR), as well as the chimera (RMMM), failed to bind MCP-1 with high affinity (Fig. 3A). To determine if RRRR or RMMM bound MCP-1 with a lower affinity, we examined the ability of higher concentrations of unlabeled MCP-1 to compete with a fixed concentration of 125I-labeled MCP-1 for binding. As shown in Fig. 3C, MCP-1 did bind specifically to RMMM, but with a significantly lower affinity (Kd = 3.5 nM) as compared to chimeras with the amino terminus of the wild-type receptor. There was minimal binding to RRRR and no specific binding to untransfected HEK-293 cells. We conclude from these data that the amino-terminal extension of the MCP-1 receptor is necessary for high affinity binding of MCP-1 and that MCP-1 also interacts with a second site on the receptor with lower affinity.
In contrast, the amino terminus of the RANTES/MIP-1α receptor was not important for high affinity binding of MIP-1α. Radiolabeled MIP-1α bound with high affinity to the wild-type receptor (RRRR, $K_d = 0.58$ nM), as well as to the construct that exchanged the receptor’s amino terminus (MRRR, $K_d = 0.69$ nM) (Fig. 4). There was no detectable specific binding (in either direct or in competition binding assays) to the wild-type MCP-1 receptor (MMMM) or to the construct that substituted the amino terminus of the RANTES/MIP-1α onto the MCP-1 receptor (RMMM). We conclude from these data that one or more of the extracellular loops, but not the amino terminus, of the RANTES/MIP-1α receptor is necessary for high affinity binding.

As a further test of the hypothesis that MCP-1 and MIP-1α bind to different regions of their respective receptors, we took advantage of the fact that the chimera MRRR bound both ligands with high affinity (Figs. 3 and 4). In competition studies, unlabeled MCP-1, but not MIP-1α, blocked the binding of radiolabeled MCP-1 to MRRR (Fig. 5). Similarly, unlabeled MIP-1α, but not MCP-1, blocked the binding of radiolabeled MIP-1α to MRRR. These data strongly suggest that MCP-1 and MIP-1α bind to different regions of MRRR and are consistent with MCP-1 binding to the amino terminus and MIP-1α binding to one or more of the extracellular loops of their respective receptors.

Role of the Amino-terminal Extension in Receptor Signaling—We have shown previously that MCP-1RB and the RANTES/MIP-1α receptor couple via Gαi to inhibit adenylyl cyclase and lower intracellular levels of cAMP (35). In the next series of experiments, we examined the ability of the chimeric receptors to mediate signal transduction in response to MCP-1 and MIP1α. The wild-type MCP-1 receptor signaled well in response to MCP-1 ($IC_{50} = 0.2$ nM), as expected (Fig. 6 and Ref. 35). There was little or no response of the RANTES/MIP-1α receptor (RRRR) to MCP-1, in agreement with published results (35). Substitution of the amino terminus of the MCP-1 receptor onto the RANTES/MIP-1α receptor, however, resulted in a dramatic increase in response to MCP-1 (compare MRRR, $IC_{50} = 7.4$ nM to RRRR, $IC_{50} = 100$ nM) (Fig. 6). Similarly, substitution of the amino terminus of the MCP-1 receptor resulted in a greater than 30-fold loss in MCP-1 responsiveness (compare chimera RMMM, $IC_{50} = 7.7$ nM to MMMM, $IC_{50} = 0.2$ nM) (Fig. 6). These data are consistent with a critical role for the amino-terminal extension of the MCP-1 receptor in ligand binding and signal transduction and further suggest that the low affinity binding of MCP-1 to RMMM is sufficient to mediate signaling, albeit at higher ligand concentrations.

In contrast, the amino terminus of the RANTES/MIP-1α receptor was not essential for receptor signaling. In response to MIP-1α, signaling of the chimera MRRR was reduced only 2–3-fold as compared to the wild-type receptor (Fig. 7: MRRR, $IC_{50} = 0.26$ nM; RRRR, $IC_{50} = 0.11$ nM). Little or no inhibition of adenylyl cyclase was mediated by RMMM or MMMM in response to MIP-1α (Fig. 7). Similar results were obtained when mobilization of intracellular calcium was assayed as a measure of signaling, or when RANTES was used instead of MIP-1α (data not shown). We conclude that the amino-terminal

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**Fig. 3.** Binding of 125I-labeled MCP-1 to wild-type and chimeric receptors. Radiolabeled MCP-1 was incubated with HEK-293 cells stably expressing the wild-type MCP-1 (MMMM) and C-C CKR-1 (RRRR) receptors, as well as with chimeras (MRRR and RMMM) that exchanged the amino-terminal extensions. Binding was determined as described under “Materials and Methods.” A, binding isotherms. Specific binding (total binding minus nonspecific binding in the presence of 100-fold excess unlabeled MCP-1) is shown for the wild-type and chimeric receptors. Nonspecific binding varied between 10 and 20% of total binding. The data shown are typical of three independent experiments.

B, Scatchard plots for MCP-1 binding to MMMM and MRRR. The $K_d$ values (MMMM, $0.33\pm0.12$ nM; MRRR, $0.27\pm0.10$ nM) were determined using the program LIGAND (34), and are within experimental error. C, competitive inhibition of 125I-labeled MCP-1 binding to MMMM and RRRR. Transfected HEK-293 cells were incubated with 1.5 nM 125I-labeled MCP-1 and the indicated concentrations of unlabeled MCP-1. The observed $K$ values were 3.5 nM for MMMM and 5.0 nM for RRRR. ○, MMMM; ●, MRRR; □, RRRR; △, RMMM.
extension of the RANTES/MIP-1α receptor is not critically involved in ligand binding or receptor signaling.

Contribution of the Extracellular Loops to MCP-1 Binding and Receptor Activation—Four additional constructs that interchanged the amino terminus plus the first extracellular loop (RRMM and MRRR, see Fig. 1) or the amino terminus plus the first two extracellular loops (RRRM and MMMR) were examined for their ability to bind and signal in response to MCP-1 and MIP-1α. Binding experiments with radiolabeled MCP-1 confirmed that the amino-terminal extension was responsible for virtually all of the high affinity binding (Table I). Furthermore, there was no detectable binding of MCP-1 to RRMM or RRRR in either direct or competition binding experiments (Table I).

The results of signaling experiments suggested that each of the three extracellular loops contributed to the agonist-dependent inhibition of adenyl cyclase by MCP-1. Thus, the chimera MMRR, which contained the amino terminus and first extracellular loop, had an IC50 of 1.4 nM, whereas MMMR, which contained the amino terminus plus the first and second extracellular loops, had an IC50 of 0.4 nM (Fig. 7), despite its relatively low expression level at the cell surface (Fig. 2). As previously shown in Fig. 6, the chimera MRRR (with all three loops substituted) was the least active of this series of constructs, and the wild-type receptor (MMMM), which contained all three extracellular loops, had an IC50 of 0.2 nM. The chimeras RRRM and RRRR, which lacked the first and second extracellular loops, respectively (as well as the amino-terminal extension), failed to signal (Fig. 8), although each of these constructs was expressed at least as well as the wild-type receptors at the cell surface. These results are summarized in Table I.

Contribution of the Extracellular Loops to MIP-1α Binding and Receptor Activation—In contrast to the MCP-1 receptor in which each of the three loops as well as the amino-terminal extension contributed to signal transduction, the third extracellular loop of the RANTES/MIP-1α receptor was especially critical for signaling. Virtually no inhibition of adenyl cyclase could be detected using the chimera RRRR, whereas MMMR, which contained only the third extracellular loop and cytoplasmic tail, did signal in response to MIP-1α (IC50 = 2.0 nM, Fig. 9). In the presence of the second and third extracellular loops (MMRR), signaling was enhanced 4-fold (IC50 = 0.46 nM). As shown earlier in Fig. 7, the addition of the first extracellular loop to this construct (MRRR, IC50 = 0.26 nM) resulted in a receptor that signaled almost as well as the wild-type C-C CKR-1. These results are summarized in Table I. We conclude from these data that in the presence of the third extracellular loop, each of the three loops of the RANTES/MIP-1α receptor contributes to signal transduction. In the absence of the third loop, however, no signaling was detected.

Binding studies confirmed the critical role of the third extracellular loop in the RANTES/MIP-1α receptor. Radiolabeled MIP-1α bound to the chimera MMMR (Kd = 24 nM), but not to RRRR (Fig. 10). Thus, the presence of the third extracellular loop alone was sufficient to support ligand binding. The presence of the second and third extracellular loops enhanced binding approximately 6-fold as compared to the third loop alone (MMRR, Kd = 41 nM versus MMMR, Kd = 24 nM), and the presence of all three loops (MRRR, Fig. 4A) resulted in high affinity binding (Kd = 0.69 nM) virtually equivalent to that of the wild-type receptor (RRRR, Kd = 0.58 nM). These data, summarized in Table I, are in sharp contrast to those obtained with the MCP-1 receptor, in which the amino terminus or all three of the extracellular loops were required for specific binding.

DISCUSSION

Activation of leukocytes by MCP-1 and related chemokines is initiated by high affinity binding to G protein-coupled, seven-
transmembrane domain receptors. A number of receptors for chemokines and other chemotactic peptides have been cloned, and there has been considerable interest in identifying functional domains. To identify ligand binding and signaling domains of the MCP-1 receptor, we took advantage of the high degree of sequence identity between the MCP-1 receptor and the RANTES/MIP-1α receptor. These two C-C chemokine receptors are 51% identical overall, but are quite divergent in their extracellular domains. We constructed a series of chimeric receptors in which the amino-terminal extension and each of the three extracellular loops of the RANTES/MIP-1α receptor were progressively substituted into the MCP-1 receptor, and vice versa. Addition of the Flag epitope at the extreme amino terminus of each of these chimeras facilitated quantitation of receptor expression, and allowed selection of stable cell lines with comparable expression at the cell surface. In this paper, we report that the amino-terminal extension of the recently cloned MCP-1 receptor is necessary for high affinity binding of MCP-1. The amino terminus was not absolutely required for signaling, however, suggesting that low affinity interactions with one or more extracellular loops (or transmembrane domains) directly mediates receptor activation and signaling. In contrast, the third extracellular loop, but not the amino terminus of the closely related RANTES/MIP-1α receptor, was required for ligand binding and signal transduction. We conclude from these data that the amino terminus of the MCP-1 receptor, but not that of the RANTES/MIP-1α receptor, is critically involved in ligand binding and receptor activation.

Two-step Mechanism of MCP-1 Receptor Activation

![Graph A](image1)

**Fig. 5.** Binding to 125I-labeled MIP-1α and MCP-1 to MRRR. A, Radiolabeled MIP-1α; B, radiolabeled MCP-1. The data shown are typical of three independent experiments.

![Graph B](image2)

**Fig. 6.** Signaling by the wild-type and chimeric receptors with exchanged amino termini in response to MCP-1. Transfected HEK-293 cells expressing the wild-type and chimeric receptors were labeled with [3H]adenine and stimulated with forskolin in the presence or absence of MCP-1. [3H]AMP pools were measured as described under "Materials and Methods." The data shown are the means of three independent experiments, and IC50 values are shown in Table I. ■, MMMM; □, MRRR; ●, RRRR; ○, RMMM.

![Graph C](image3)

**Fig. 7.** Signaling by the wild-type and chimeric receptors in response to MIP-1α. Inhibition of adenylyl cyclase was assayed as described in Fig. 6. The data shown are the means of three independent experiments, and IC50 values are shown in Table I. ■, MMMM; □, MRRR; ●, RRRR; ○, RMMM.

**TABLE I**

| Receptor | IC50 (nM) | Kd (nM) | Bmax (fmol/10^6 cells) |
|----------|-----------|---------|------------------------|
| MMMM | 0.2 (0.14) | ND | 0.33 (0.12) ND 2.8 (1.3) |
| MMRM | 0.4 (0.1) | 1.97 (0.9) | 0.36 (0.17) 24.0 (4.7) 1.1 (0.2) |
| MRRR | 1.4 (1.2) | 0.46 (0.14) | 0.28 (0.10) 4.1 (2.6) 4.2 (1.4) |
| MRRR | 7.4 (1.3) | 0.26 (0.12) | 0.27 (0.10) 0.69 (0.24) 4.9 (1.8) |
| MRRR | >100 | 0.11 (0.06) | 5.00 (1.5) 0.58 (0.10) 3.9 (1.9) |
| RRRM | ND | ND | ND ND |
| RRRM | ND | ND | ND |
| RRRM | 7.7 (1.3) | 3.50 (0.98) | ND 4.2 (1.8) |

* ND, not detectable.
initial studies with these constructs revealed that high affinity binding of MCP-1 correlated with the presence of the 40-residue amino terminus of the MCP-1 receptor. Signaling data obtained in transfected HEK-293 cells also supported a critical role for the amino terminus. Thus, substitution of the wild-type amino terminus of the MCP-1 receptor resulted in a greater than 30-fold increase in the IC50 for inhibition of adenylyl cyclase (compare RMMM and MMMM, Fig. 6 and Table I). That the chimera RMMM signaled in response to MCP-1 indicated that high affinity binding to the wild-type amino terminus was not absolutely required for receptor activation, and also suggested that MCP-1 bound to a second domain of the receptor to initiate signaling. Additional studies revealed low affinity binding of MCP-1 to RMMM, consistent with the higher IC50 for signal transduction. To determine if this low affinity binding was to one or more of the extracellular loops, versus the substituted amino terminus (i.e. to the "MMM" versus the "R" portion of the RMMM chimera), we performed additional binding studies using the chimeras RRM and RRMM. We were unable to demonstrate specific binding of MCP-1 to either of these chimeras, though both were expressed well at the cell surface. We conclude, therefore, that signaling of the MCP-1 receptor can be mediated by low affinity binding of MCP-1 to one or more of the three extracellular loops.

The presence of all three of the extracellular loops of the MCP-1 receptor appeared to be required for optimal signaling. Thus, loss of the third extracellular loop (MMMR) decreased signaling by 2-fold, loss of the second and third loops (MMRR) decreased signaling by an additional 3-fold, and loss of all three extracellular loops (MRRR) decreased signaling an additional 5-fold (Table I). Thus with regard to the MCP-1 receptor, the first extracellular loop may be particularly important for receptor activation.

It is possible that the receptor transmembrane domains contribute to the binding of MCP-1. However, there is greater than 70% identity at the amino acid level between the MCP-1 and the RANTES/MIP-1α receptor transmembrane domains, making it unlikely that these regions contribute to specificity. Furthermore, while agonist interactions with residues in the transmembrane domains are typical in the case of small molecules, these interactions are unusual for peptides exceeding 3 amino acids in length. Thus, the opiates (δ, κ, and μ), tachykinins, endothelin, and the agonist peptide of the thrombin receptor have all been shown to interact with the extracellular loops of their respective seven-transmembrane domain receptors (reviewed in Ref. 17).

A model that incorporates the results of these experiments is shown in Fig. 11. In this model, the high affinity binding of...
Two-step Mechanism of MCP-1 Receptor Activation

MCP-1 (either in monomorphic or dimeric form) to the amino-terminal extension serves to position MCP-1 for a lower affinity interaction with a second site on the receptor that ultimately initiates signaling. The model predicts that in the absence of high affinity binding to the amino terminus, signaling would still be possible, but would require higher MCP-1 concentrations. This is precisely what was found for the chimera RMMR; the dose-response curve for inhibition of adenylyl cyclase was shifted to the right. Signaling by the chimera MRRR is also consistent with this model. In this case, the parent receptor(RRRR), signals well in response to nanomolar concentrations of MIP-1α, RANTES, and MCP-3 (35, 37), but requires significantly higher concentrations of MCP-1 (IC₅₀ > 0.1 µM). Substitution of the amino terminus of the MCP-1 receptor onto the RANTES/MIP-1α receptor to produce MRRR resulted in a chimera that bound MCP-1 with high affinity, and signaled in response to nanomolar concentrations of MCP-1 (i.e. this chimera exhibited a gain of function as compared to the parent receptor, RRRR). The most parsimonious explanation of these data is that the high "local" concentration of MCP-1 achieved by tethering it to the amino terminus allows a more efficient interaction with the extracellular loops, and hence causes activation of this chimera.

An analogous signaling mechanism has been described for the human thrombin receptor, whose amino-terminal extension is cleaved by thrombin to expose a truncated amino terminus, which then interacts with distal parts of the receptor as a "tethered ligand" (38). MCP-1 appears to use a modification of this mechanism in which the receptor amino terminus binds the peptide ligand with high affinity, effectively creating a "pseudo-tethered" ligand. It has been proposed, in this regard, that the high affinity binding of leukotriene hormone to the amino terminus allows interaction with a charged residue in the first extracellular loop of its receptor (22). Similarly, C5a appears to interact with the amino terminus, as well as a second site on the C5a receptor (30, 31). A recent study with the IL-8R reports that multiple regions of the receptor are involved in binding IL-8 (29).

In sharp contrast to the MCP-1 receptor amino terminus, the amino-terminal 32 residues of the RANTES/MIP-1α receptor do not appear to be important in ligand binding or receptor activation. A chimera with a substituted amino terminus (MRRR) bound MIP-1α with virtually the same affinity as the wild-type receptor (RRRR). Similarly, signal transduction in response to MIP-1α was comparable in the presence of the wild-type (RRRR) or the substituted (MRRR) amino terminus. The presence of the third loop alone (chimera MMMR) was sufficient for binding and signaling in response to MIP-1α, despite the relatively low surface expression of this chimera. These results are very different from those obtained with the MCP-1 receptor, in which the presence of the third extracellular loop alone (RRRM) did not result in detectable binding or signaling. Our data suggest that in the case of the RANTES/MIP-1α receptor, the third extracellular loop is the critical domain of the receptor. Chimeras that lacked this third loop (e.g. RMMM, RMRR, or RRRR) neither bound nor signaled in response to MIP-1α, despite their high level of surface expression as compared to MMMR.

In summary, we have created chimeric MCP-1 and RANTES/MIP-1α receptors and correlated their ligand binding and signaling properties. Our data suggest a two-step model leading to activation of the MCP-1 receptor. In the first step, MCP-1 binds with high affinity to the amino-terminal extracellular domain (amino acids 1-40) of the receptor. The second step is a low

![Fig. 10. Role of the third extracellular loop of C-C CKR-1 in binding MIP-1α.](image)

![Fig. 11. Hypothetical model for activation of the MCP-1 receptor.](image)
affinity interaction between the "pseudo-tethered" MCP-1 and one or more of the receptor's extracellular loops, and serves to initiate signal transduction. The mechanism of ligand binding and activation of the MCP-1 receptor thus incorporates features of both large glycoprotein and small peptide interactions with seven-transmembrane domain receptors. In contrast, ligand interaction between the "pseudo-tethered" MCP-1 and the "molecularly clamped" fMLP receptor is dependent upon the third extracellular loop, and not the amino terminus. In comparison to other chemoattractant receptors, ligand interactions with the MCP-1 receptor are most similar to the IL-8 and C5a receptors. The RANTES/MIP-1α receptor, however, interacts with its ligands in a manner reminiscent of the binding of fMLP to the fMLP receptor in which multiple domains are required for high affinity binding. These data thus suggest significant functional differences between these two highly homologous C-C chemokine receptors. The identification of ligand binding and signaling domains provides an important starting point for the development of receptor agonists and antagonists. Such reagents are likely to prove useful in the treatment of a wide range of human diseases characterized by prominent monocyte/macrophage infiltrates.

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