The Amino-terminal Domain of CCR2 Is Both Necessary and Sufficient for High Affinity Binding of Monocyte Chemoattractant Protein 1

RECEPTOR ACTIVATION BY A PSEUDO-TETHERED LIGAND

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High affinity binding of monocyte chemoattractant protein 1 (MCP-1) requires the presence of the amino-terminal domain of CCR2, the MCP-1 receptor. Here we report that the 35 amino-terminal residues of CCR2, expressed as a membrane-bound fusion protein, bound MCP-1 with an affinity similar to that of the intact, wild-type receptor. Furthermore, the amino-terminal fusion protein enhanced, in trans, agonist-dependent activation of a CCR2 variant that was engineered to lack the high affinity binding sites for MCP-1. Mutation of highly conserved cysteines in the amino-terminal domain and third extracellular loop of CCR2, but not in the fusion protein, resulted in a dramatic loss of MCP-1 binding, suggesting the existence of a critical intramolecular disulfide bond that positions the amino-terminal protein for ligand interaction. These data indicate that the amino-terminal region of CCR2 is both necessary and sufficient for the high affinity binding of MCP-1 and provide the first direct evidence for activation of a chemokine receptor by a pseudo-tethered ligand. In this model, high affinity binding by the relatively short amino-terminal domain of CCR2 serves to tether MCP-1 and enhance low affinity interactions with distal regions of the receptor.

Monocyte chemoattractant protein 1 (MCP-1) is a 76-amino acid peptide and a member of a family of potent leukocyte agonists known as the chemokines (1–4). The responses of chemokines are mediated by seven-transmembrane domain, G-protein-coupled receptors, several of which have been recently identified as cofactors in the internalization of the G-protein-coupled receptors, are not well understood. We have recently shown, in the context of a complete receptor, that the amino-terminal region and Cys-277 in the third extracellular loop of CCR2 were mutated to alanine by overlapping PCR (16). cDNA constructs were subcloned into pcDNA3 and transfected into human embryonic kidney (HEK)-293 cells to produce stable cell lines as described (19). Cell-surface expression was quantitated by enzyme-linked immunosorbent assay (ELISA) using the anti-Flag antibody M1 (IBI/Kodak) (12).

EXPERIMENTAL PROCEDURES

Reagents—Recombinant chemokines were obtained from R&D Systems, Inc. (Minneapolis, MN). LipofectAMINE and G418 sulfate were from Life Technologies, Inc. Restriction enzymes were from Boehringer Mannheim. All other reagents were obtained from Sigma.

cDNA Constructs and Transfections—CCR2B was cloned as described previously (11) and is referred to throughout as CCR2. The epitope-tagged (12) amino-terminal extensions of CCR2 (residues 2–36) (11) and CCR1 (residues 2–54) (13) were produced by PCR and fused to the human CD6 transmembrane domain and cytoplasmic tail (kindly provided by Dr. Shaun Coughlin, University of California, San Francisco) at the EcoRV site, as described by Chen et al. (14), to create CCR2(2–36)/CD8 and CCR1(2–34)/CD8, respectively. A PCR product containing 40 residues of the MCP2 amino terminus was fused at the Nhel site in the murine erythropoietin receptor (Epo-R) (15) (kindly provided by Dr. Mark Goldsmith, Gladstone Institute of Virology and Immunology, San Francisco) to create CCR2(2–41)/Epo-R. These restriction sites were chosen to conserve the native CCR2 sequence. Cysteine 32 in the amino-terminal region and Cys-277 in the third extracellular loop of CCR2 were mutated to alanine by overlapping PCR (16). cDNA constructs were subcloned into pcDNA3 and transfected into human embryonic kidney (HEK)-293 cells to produce stable cell lines as described (17). Transient transfections were performed with HEK-293T, a cell line expressing the polyoma virus large T antigen (18) (kindly provided by Dr. Mark Goldsmith), as described (19). Cell-surface expression was quantitated by enzyme-linked immunosorbent assay (ELISA) using the anti-Flag antibody M1 (IBI/Kodak) (12).

Chemokine Binding Assay—Binding was performed with 125I-labeled MCP-1 as described (20). The dissociation constant (Kd) and maximum bound (Bmax) were determined with the computer program LIGAND (21).

Adenylyl Cyclase Assays—HEK-293 cell lines expressing the chimera 1222 (10) or CCR1 were transiently transfected with the indicated cDNAs using LipofectAMINE. Inhibition of adenylyl cyclase was assayed as described (17). The cells were stimulated by addition of fresh medium containing 1 mM 3-isobutyl-1-methylxanthine and either chemokine alone, forskolin alone (10 μM to activate adenylyl cyclase), or chemokine plus forskolin for 20 min at room temperature. Data are expressed as a percent of the maximal stimulation achieved by forskolin alone. All data points were determined in duplicate. Half-maximal inhibition (IC50) was determined with a curve-fitting program, PRISM (GraphPad, San Diego, CA). One-way analysis of variance was used to determine p values.
**RESULTS**

We have recently demonstrated that the amino-terminal domain of CCR2 plays a critical role in ligand binding and receptor activation (10). To determine if this short amino terminus could account for all of the high affinity binding, we created the chimeric construct CCR2(2–36)/CD8, in which the 35 amino-terminal residues of CCR2 were fused to the transmembrane domain and cytoplasmic tail of human CD8. In equilibrium binding assays, [125I]-labeled MCP-1 bound as well to CCR2(2–36)/CD8 as to the wild-type MCP-1 receptor (Fig. 1). Similar results were obtained when the amino-terminal region of CCR2 was fused to the transmembrane domain of the Epo-R (CCR2(2–41)/Epo-R). Scatchard analysis revealed virtually identical $K_d$ values of 680, 640, and 620 pM, respectively, for the wild-type receptor and the CCR2(2–36)/CD8 and CCR2(2–41)/Epo-R constructs (Fig. 1). In contrast, little or no binding of MCP-1 was detected when the amino-terminal region (amino acids 2–34) of CCR1, the closely related receptor for the chemokines RANTES (regulated on activation normal T cell expressed and secreted) and macrophage inflammatory protein-1α (13), was fused to CD8. Analysis of these constructs by ELISA revealed comparable levels of cell-surface expression, determined in triplicate. Similar results were obtained with receptors that were epitope-tagged with the hemagglutinin antigen (31) instead of the Flag antigen (data not shown). The vertical bars denote standard deviation. Shown is one of three similar experiments. Nonspecific binding was typically less than 10% of total bound.

![Figure 1](https://example.com/figure1.png)

**FIG. 1. Specific binding of [125I]-MCP-1 to CCR2, CCR2(2–36)/CD8, CCR2(2–41)/Epo-R, and CCR1(2–34)/CD8.** Nonspecific binding, determined by including a 100-fold excess of unlabeled MCP-1, was subtracted from the total cpm bound. Scatchard analysis of binding data is presented in the inset ($B/F = \text{bound/free}$). All data points were determined in triplicate. Similar results were obtained with receptors that were epitope-tagged with the hemagglutinin antigen (31) instead of the Flag antigen (data not shown). The vertical bars denote standard deviation. Shown is one of three similar experiments. Nonspecific binding was typically less than 10% of total bound.

**TABLE I**

**Expression of the MCP-1 receptor amino terminus on the surface of HEK-293 cells**

| Constructs | ELISA $^a$ | $K_d$ $^a$ | $B_{\text{max}}$ $^a$ |
|------------|------------|-------------|-----------------------|
| HEK-293 untransfected | 0.11 ± 0.04 | ND | ND |
| CCR2B | 0.40 ± 0.2 | 680 | 28,715 |
| CCR2(2–36)/CD8 | 0.37 ± 0.03 | 640 | 24,431 |
| CCR2(2–41)/Epo-R | 0.36 ± 0.02 | 620 | 24,417 |
| CCR1(2–34)/CD8 | 0.31 ± 0.1 | ND | ND |

$^a$ Values represent the mean ± S.D.

**FIG. 2. A model illustrating CCR2 activation in trans.** The amino terminus of the CCR2 fused to CD8 as a membrane anchor domain (CCR2(2–36)/CD8) binds MCP-1 with high affinity. The tethered ligand is positioned to contact low affinity binding sites on the chimeric receptor 1222 or CCR1 to activate signaling, indicated by the jagged arrow. Data are presented in Fig. 3. Interactions between the transmembrane domain of CD8 and the receptor are not implied.

Although virtually all of the high affinity binding could be attributed to the 35 amino-terminal residues of CCR2, earlier work from our laboratory has demonstrated that low affinity interactions of MCP-1 with distal regions of the receptor are required for signal transduction (10). Taken together, these results suggested a model of receptor activation in which noncovalent “tethering” of MCP-1 by the receptor amino terminus enhances the low affinity interactions that lead to G-protein activation (Fig. 2). To test this model, we asked whether coexpression of the CCR2(2–36)/CD8 construct would complement activation of a mutant form of CCR2 that was modified to lack the high affinity binding site for MCP-1 (see Fig. 2 for a drawing depicting the experimental design). The construct 1222 is a variant of CCR2 in which the amino-terminal region has been replaced with the corresponding region of CCR1; the three extracellular/intracellular loops and associated transmembrane domains (222) are from CCR2. We have shown previously (10) that this chimera (originally designated RMMM) failed to bind MCP-1 with high affinity and that signaling in response to MCP-1 was reduced by 97% (10). Coexpression of CCR2(2–36)/CD8 with 1222 significantly enhanced signaling as compared with 1222 alone (IC50 of adenylyl cyclase, 13 versus 4 nM; $p < 0.001$) (Fig. 3A). In contrast, coexpression of CCR1(2–34)/CD8, which did not bind MCP-1 (Fig. 1), did not enhance signaling by 1222. No signal was observed with CCR2(2–36)/CD8 alone.

As a further test of this model, we performed cotransfection experiments with the CCR2(2–36)/CD8 construct and wild-type CCR1. CCR1 signaled, albeit poorly, in response to high concentrations of MCP-1 (Fig. 3B) (10, 17). Coexpression of CCR2(2–36)/CD8 with CCR1 significantly enhanced MCP-1-dependent signaling ($p < 0.001$). In fact, in this experiment, the degree of transactivation was comparable to that achieved by transfection of wild-type CCR2. As expected, cotransfection of CCR1(2–34)/CD8 did not enhance signaling. These data provide direct evidence for activation of the receptor in trans by a tethered form of MCP-1.

CCR2 contains cysteines in the amino-terminal region at position 32 (Cys-32) and in the third extracellular loop at position 277 (Cys-277) that are conserved in all chemokine
receptors and that may contribute to maintenance of receptor conformation through the formation of a disulfide bond. To investigate their role in ligand binding and signaling, we changed each of these two residues to alanines. Mutation of either residue resulted in a complete loss of binding activity (Fig. 4A), although receptor expression on the cell surface was comparable (Table II). In contrast, mutation of Cys-32 in CCR2(2–36)/CD8 did not significantly affect binding (Fig. 4B), suggesting that this amino acid did not interact directly with MCP-1. Finally, we determined the effect of mutation of these cysteines on receptor signaling. Mutation of either Cys-32 or Cys-277 significantly impaired MCP-1-induced signaling (Fig. 5). Loss of this putative disulfide bond led to a greater impairment of signaling than could be accounted for by the lack of high affinity binding of MCP-1 alone (compare with chimera 1222).

**TABLE II**

| Construct | Binding $^a$ $K_d$ | Surface expression $^b$ |
|-----------|-------------------|------------------------|
| HEK-293T  | ND                | 0.17 ± 0.05            |
| CCR2B     | 0.42 ± 0.14       | 0.98 ± 0.18            |
| CCR2(C32A)| ND                | 0.99 ± 0.13            |
| CCR2(C277A)| ND                | 0.83 ± 0.24            |
| CCR2(2–36)/CD8 | 0.50 ± 0.26 | 0.80 ± 0.17 |
| CCR2(2–36; C32A)/CD8 | 0.36 ± 0.26 | 0.62 ± 0.21 |

$^a$The values represent the mean ± S.D. of four experiments.

$^b$The values represent the mean ± S.D. of eight experiments.

FIG. 3. **Inhibition of adenyl cyclase activity by MCP-1.** A, HEK-293 cells stably expressing 1222 were transiently transfected with CCR2(2–36)/CD8 or CCR1(2–34)/CD8. MCP-1-induced inhibition of forskolin-stimulated adenyl cyclase activity was assayed by measuring cAMP levels as described (17). Each data point was determined in duplicate. The arrow denotes a leftward shift in the dose-response curve, representing a decrease in the half-maximal response to MCP-1. Shown is one of three similar experiments. B, HEK-293 cells stably expressing CCR1 were transiently transfected with CCR2(2–36)/CD8 or CCR2. The transfection efficiency, determined at 48 h with a β-galactosidase cDNA, was 60–80%. The ratio of transiently expressed chimeras to 1222 (or the CCR1), determined by ELISA, was 3–5. Vertical bars denote standard deviation. The $p$ values were determined by one-way analysis of variance ($n = 3$).

FIG. 4. **Effect of the mutation of cysteines in CCR2 and CCR2(2–36)/CD8 on MCP-1 binding.** A, competition binding of 125I-MCP-1 to wild-type CCR2 or CCR2 in which either the amino-terminal cysteine (C32A) or the third extracellular loop cysteine (C277A) was changed to alanine. B, binding of 125I-MCP-1 to the CCR2 amino-terminal domain fused to CD8 (CCR2(2–36)/CD8) or to the same construct in which the cysteine was changed to alanine (C32A). Wild-type CCR2 was included as an internal control. All constructs were analyzed after transient transfection in HEK-293T cells. The error bars represent the standard error of the mean. Shown is one of three similar experiments.

MCP-1 Activation by Tethered MCP-1
amino-terminal domain, however, thrombin cleaves this region of the thrombin receptor (24). After binding to its receptor appears to be mediated by the long amino-terminal exodomain motif of CCR2 in ligand binding and receptor activation. As with the glycoprotein hormone receptors and the thrombin receptor (i.e. high affinity binding of ligand to the amino terminus to produce a tethered ligand). Unlike the thrombin receptor, however, CCR2 binds its ligand (MCP-1) non-covalently, and thus its activation is mediated by a “pseudo-tethered” ligand and also requires the presence of the ligand. A prediction of this model, as yet unproven, is that two distinct domains of MCP-1 interact with CCR2: one for the high affinity binding to the amino terminus and one for interacting with distal regions of the receptor to trigger activation.

Four highly conserved cysteines are present in the extracellular regions of all chemokine receptors. Two of these cysteines (located in the first and second extracellular loops) are a constant feature of seven-transmembrane domain receptors and, in the case of rhodopsin, have been shown to participate in a disulfide bond (25). In a number of seven-transmembrane receptors, mutation of either of these cysteines results in little or no expression of the receptor at the cell surface, presumably due to perturbations in the tertiary structure of the protein (25). The other two cysteines are found in the amino-terminal region (position 32 in CCR2) and in the third extracellular loop (position 277); the exact conservation of these amino acids in both CC and CXC chemokine receptors, but not all G-protein-coupled receptors, suggests that they form a disulfide bond critical for receptor-chemokine interactions. Data presented here support this hypothesis and further indicate that MCP-1 does not interact directly with the amino-terminal cysteine, as mutation of this residue in the CD8 construct did not impair binding.

There are several other examples in which a putative disulfide bond between the amino-terminal domain and the third extracellular loop has been investigated for its importance in the binding of either peptides or lipids to seven-transmembrane domain receptors. Unlike our findings with CCR2, loss of this bond in the bradykinin B2 receptor had no effect on ligand binding (26). A similar lack of effect on ligand binding was seen upon mutation of analogous cysteines in the thromboxane A2 receptor (27). In the angiotensin II receptor, however, mutation of either of the two analogous cysteines reduced ligand-binding affinity by 90% (28). Interestingly, these mutations had no effect on the binding of a non-peptide ligand (losartan) to the angiotensin II receptor (28). Finally, Leong et al. (29) found that mutation of either of the two analogous cysteines in CXCR1 led to a marked impairment in ligand-binding affinity and receptor activation. Taken together with the data in this paper, these observations suggest that an important role for an intramolecular disulfide bond in optimizing the binding of chemotactic peptides, but not small molecule agonists, to seven-transmembrane segment receptors. In the case of CCR2, the lack of signaling in the Cys-32 and Cys-277 mutants, even as compared with a mutant (1222) that lacks high affinity binding of MCP-1, further supports the notion that this disulfide bond restrains the receptor in a conformation that allows activation upon ligand binding. It will be interesting to determine if this paradigm also applies to chemokine receptors in which the amino-terminal region is not critically involved in ligand binding, such as CCR1 (10) or CCR5 (30).

In summary, we have shown that MCP-1 binds to the 35-amino acid amino-terminal domain of CCR2 with an affinity indistinguishable from that of the wild-type receptor. To our knowledge, this is the first demonstration that the binding activity of a G-protein-coupled receptor can be fully recapitulated by the long amino-terminal segment. We have also
demonstrated in trans activation of altered forms of CCR2 by the binding of MCP-1 to this short amino-terminal domain. These experiments have provided significant new data that support the two-site, two-step model of CCR2 activation proposed in our earlier publication (10). This model may be highly relevant to the mechanism of activation of glycoprotein hormone and other chemotactic peptide receptors. Finally, we have shown that mutation of two highly conserved cysteines in the amino-terminal domain and third extracellular loop abolished ligand binding in the wild-type receptor, whereas mutation of this cysteine in the amino-terminal/CD8 construct had no effect on binding. These data provide evidence for a critical disulfide bond that positions the amino-terminal region of CCR2, and, by analogy, other chemokine receptors as well, for ligand binding. Studies designed to identify amino acids within the amino-terminal region of CCR2 that directly interact with MCP-1 are currently in progress.

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