Role of the First Extracellular Loop in the Functional Activation of CCR2

THE FIRST EXTRACELLULAR LOOP CONTAINS DISTINCT DOMAINS NECESSARY FOR BOTH AGONIST BINDING AND TRANSMEMBRANE SIGNALING*

(Received for publication, June 8, 1999, and in revised form, August 18, 1999)

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The first extracellular loop of CCR2 is one of the principal determinants of the functional complex that allows the formation of the functional CCR2-G protein complex.

The recruitment of leukocytes to sites of inflammation is initiated by locally produced chemotactic cytokines also called chemokines that interact with specific chemokine receptors. Chemokines are a rapidly growing family of small proteins that share a homology in their primary structure and are characterized by their ability to recruit and activate various types of leukocytes (1). Based on the position of the first two cysteine residues, they are subdivided into several subfamilies. In the α- or CXC subfamily, the first two cysteines are separated by an amino acid residue that is missing in the β- or CC subfamily. Lymphotactin lacks some of the cysteine residues and may be the first identified member of a new chemokine subfamily (2). A fourth human chemokine type, called fractalkine, was recently identified (3). Unlike other chemokines, fractalkine consists of a chemokine domain attached to a mucin-like extension that can function as a membrane anchor. Monocyte chemotactic protein 1 (MCP-1) belongs to the β-subfamily and is a potent chemotaxant for monocytes, basophils, and certain subsets of T cells (4–7). It is secreted by a variety of cells in response to stimulation by cytokines and participates in the inflammatory response by binding to distinct receptors (8).

Although the magnitude of an inflammatory response is generally proportional to the concentration of MCP-1, monocyte recruitment is at least in part controlled by the level of CCR2 expression. Recent studies in our laboratory demonstrated that pro-inflammatory cytokines including MCP-1 itself rapidly down-regulated the expression of CCR2, which may aid the retention of monocytes at sites of inflammation after their recruitment from the circulation (16). These cytokines also induced the secretion of MCP-1 by monocytes and initiated the switch from the MCP-1-responsive state to the MCP-1-unresponsive state, which coincided with the loss of CCR2. In contrast, plasma levels of low density lipoproteins that are characteristic for hypercholesterolemia increased monocyte CCR2 expression. As a consequence the chemotactic activity of monocytes was enhanced, which may result in their excessive recruitment to the vessel wall in chronic inflammation and atherogenesis (17, 18).

1 The abbreviations used are: MCP-1, monocyte chemotactic protein-1; BSA, bovine serum albumin; CCR2, MCP-1 receptor; G protein, GTP-binding protein; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; GTPγS, guanosine 5′-3-O-(thio)triphosphate.

* This work was supported in part by National Institutes of Health Grants HL56989-01 (SCOR in Molecular Medicine and Atherosclerosis) and AI41719-01A1 (to O. Q.) and by the Tobacco-related Disease Research Program, California, Grant 6IT-0133 (to O. Q.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The physiological cellular responses to monocyte chemotactic protein-1 (MCP-1), a potent chemotactic and activating factor for mononuclear leukocytes, are mediated by specific binding to CCR2. The aim of this investigation is to identify receptor microdomains that are involved in high affinity agonist binding and receptor activation. The results from our functional studies in which we utilized neutralizing antisera against CCR2 are consistent with a multidomain binding model, previously proposed by others. The first extracellular loop was of particular interest, because in addition to a ligand-binding domain it contained also information for receptor activation, crucial for transmembrane signaling. Replacement of the first extracellular loop of CCR2 with the corresponding region of CCR1 decreased the MCP-1 binding affinity about 10-fold and prevented transmembrane signaling. A more detailed analysis by site-directed mutagenesis revealed that this receptor segment contains two distinct microdomains. The amino acid residues Asn104 and Glu105 are essential for high affinity agonist binding but are not involved in receptor activation. In contrast, the charged amino acid residue His100 does not contribute to ligand binding but is vital for receptor activation and initiation of transmembrane signaling. We hypothesize that the interaction of agonist with this residue initiates the conformational switch that allows the formation of the functional CCR2-G protein complex.

In analogy to the thrombin receptor (19) or the receptors for interleukin-8 CXCR1 and CXCR2 (20), the amino-terminal tail is involved in the functional activation of the receptor and in the retention of monocytes at sites of inflammation after their recruitment from the circulation (16). These cytokines also induced the secretion of MCP-1 by monocytes and initiated the switch from the MCP-1-responsive state to the MCP-1-unresponsive state, which coincided with the loss of CCR2. In contrast, plasma levels of low density lipoproteins that are characteristic for hypercholesterolemia increased monocyte CCR2 expression. As a consequence the chemotactic activity of monocytes was enhanced, which may result in their excessive recruitment to the vessel wall in chronic inflammation and atherogenesis (17, 18).

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of CCR2 appears to be a determinant for chemokine binding as well as selectivity (21). However, the binding of peptide agonists to chemotaxtactive receptors is very complex. More detailed analyses of the ligand-binding domains of CXCR1, CXCR2 (22, 23), and of the N-formyl-methionyl-leucyl-phenylalanine receptor (24, 25) suggested that multiple receptor segments are involved in the functional receptor-ligand interaction. Similarly, the interaction of MCP-1 with multiple domains of CCR2 may be necessary for intact transmembrane signaling.

In this report we describe the functional characterization of the first extracellular loop of CCR2. Our results suggest a dual role for this receptor segment. It contains a site that supports high affinity binding of MCP-1. In addition to this ligand-binding domain, we have identified a primary structure that appears essential for ligand-induced activation of G proteins without affecting the binding affinity. This study provides the first evidence that an extracellular domain of CCR2 distinct from the ligand-binding site is required for functional transmembrane signaling.

**EXPERIMENTAL PROCEDURES**

**Materials**—HEK 293 cells were purchased from American Type Culture Collection (Manassas, VA). LipofectAMINE, G418 sulfate, and all tissue culture reagents were from Life Technologies, Inc. Restriction enzymes and other reagents for the manipulation of DNA were from Roche Molecular Biochemicals, Life Technologies, Inc., and Promega Corp. (Madison, WI). Recombinant MCP-1 was purchased from R & D Systems, Inc. (Minneapolis, MN), 125I-MCP-1 (specific activity, 2200 Ci/mmol) and [35S]GTP-γ-S (1300 Ci/mmol) were from NEN Life Science Products. All other reagents were of highest purity available.

**Construction of Mutant Receptors**—The cDNAs for CCR2B and CCR1 were obtained from the human THP-1 cell line. Total RNA was isolated, reversed-transcribed, and amplified by polymerase chain reaction (PCR). Their nucleotide sequence was confirmed by DNA sequencing and reverse-transcribed, and amplified by polymerase chain reaction (PCR). The amplified cDNA, subcloned into the expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA), and the construct was analyzed by DNA sequencing. The amplified fragments were subcloned in frame into the pGEX-2T vector (Amer sham Pharmacia Biotech), sequenced, and expressed as fusion proteins in E. coli. The fusion proteins were isolated with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech).

**Intracellular Calcium Measurement**—Cells were grown to confluence in a T75 flask, washed once with PBS, and labeled with 5 μM Indo-1 AM (Molecular Probes, Inc., Eugene, OR) in RPMI 1640 without phenol red, 10 mM HEPES, 0.1% BSA, pH 7.4, for 30 min at 37 °C in the dark. The cells were then washed twice with PBS, harvested with protease-free dissociation buffer, and suspended at a density of 1.5 × 10^6 cells/ml in RPMI 1640 containing 0.5% BSA. Changes in the concentration of intracellular calcium ([Ca^2+]) in response to various concentrations of MCP-1 were monitored as described (24). Fluorescence was continuously measured simultaneously at 400 and 490 nm on an LS50B luminescence spectrophotometer (Perkin-Elmer) with the excitation set at 340 nm. The change in [Ca^2+], expressed as change of the fluorescence ratio F_400/F_490 was measured.

**Equilibrium Binding Analysis**—Binding assays were performed essentially as described previously (16). Briefly, the cells were washed with PBS and 0.3 × 10^6 cells were suspended in 100 μl of RPMI 1640 containing 0.5% BSA. The cell suspension was incubated with 0.2 nM 125I-MCP-1 and varying concentrations of unlabeled ligand for 60 min at room temperature. At the end of the incubation the cells were separated from the buffer by centrifugation through 300 μl of an equal mixture of dibutyl phthalate and disethyl phthalate (Alrich), and the pellet was washed with PBS. The pellets were associated with IgG fractions of the neutralizing antiserum AbTM7C, which inhibited receptor-mediated activation of G protein. Inclusion of AbTM7C IgG in the binding experiment inhibited receptor-mediated activation of G protein. The IgG fraction of the neutralizing antiserum AbTM7C, which is directed against the carboxyl-tail of CCR2B, was included to determine the IgG fraction of the neutralizing antiserum AbTM7C, which was directed against the carboxyl-tail of CCR2B, was included to determine.

**Intracellular Membrane Preparation**—Cells were detached from the culture flask with protease-free dissociation buffer (Life Technologies, Inc.), collected by centrifugation, and washed twice with phosphate-buffered saline (PBS). Cells were suspended in 20 μl HEPES, pH 7.4, containing 5 mM MgSO_4 and a mixture of protease inhibitors (50 units/ml aprotinin, 5 mM benzamidine, 14.5 μM pepstatin, 0.1 mM leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride), placed on ice, and homogenized. Unbound proteins and cell nuclei were removed by centrifugation at 4000 rpm for 10 min at 4 °C, and crude membranes were then isolated by centrifugation at 48,000 × g for 30 min at 4 °C. The membranes were either used immediately or stored at −80 °C.

**Generation of Antisera and Flow Cytometry**—Two antisera directed against the first (AbTM23) and second (AbTM45) extracellular loops and one against the intracellular carboxy-terminal tail (AbTM7C) of CCR2 were generated. The CCR2-specific fragments containing the amino acid residues Ile93 to His124, Pro174 to Met205, and Gly309 to Leu360, representing the first, second extracellular loops, and the carboxy-terminal tail, respectively, were amplified by PCR. The amplified fragments were subcloned in frame into the pGEX-2T vector (Amer sham Pharmacia Biotech), sequenced, and expressed as fusion proteins in E. coli. The fusion proteins were isolated with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech). The IgG fractions of the neutralizing antiserum AbTM7C, which is directed against the carboxyl-tail of CCR2B, was included to determine the IgG fraction of the neutralizing antiserum AbTM7C, which was directed against the carboxyl-tail of CCR2B, was included to determine.

**Chemotaxis Assay**—THP-1 monocytes were suspended at a concen-
Both chambers are separated by a 5-
line second (AbTM45) extracellular loops of CCR2, and IgG fractions were prepared. To test their specificity, CCR2B-transfected HEK 293 cells (Tyrode’s salt buffer (Sigma), 1% NaHCO3, 0.1% BSA, pH 7.4. The cell suspension (51 µl) was loaded into the upper chamber of the 48-well microchemotaxis Boyden chamber (Neuroprobe, Gaithersburg, MD), and 10 nM MCP-1 in chemotaxis buffer was added to the lower chamber. Both chambers are separated by a 0.5-µm pore-size polycarbonate membrane (Poretics Corp., Livermore, CA). After 1 h at 37 °C in a 5% CO2 atmosphere, the side of the membrane that was in contact with the cell suspension was washed to remove any cells. The migrated cells adhering to the underside of the membrane were fixed in 1% glutaraldehyde, stained with crystal violet, and counted in four × 400 high power fields of three replica filters. To determine the inhibitory effects of the antisera AbTM23 and AbTM45 on chemotaxis, THP-1 monocytes were first incubated with the IgG fractions of the individual antisera or with a combination of both (10 µg/ml) for 30 min at 4 °C before their placement into the Boyden chamber. Nonspecific migration was determined by including 1 µg/ml neutralizing mouse anti-human MCP-1 monoclonal antibody (R & D Systems Inc., Minneapolis, MN). All data were expressed as chemotaxis index defined as the number of cells that migrated to MCP-1 divided by the number of cells that migrated in the presence of neutralizing anti-MCP-1 antibody.

Other Analytical Analyses—Protein was determined by the method of Lowry et al. (31). Data are expressed as the mean ± S.D.

RESULTS

Inhibition of Receptor Function by Antibodies—A hypothetical model for binding of MCP-1 by CCR2 that involves the amino-terminal tail was proposed recently (32). To identify other extracellular domains that are fundamental for receptor function, we generated antisera against the first (AbTM23) and second (AbTM45) extracellular loops of CCR2. The specificity of the antisera was tested by flow cytometry, and both AbTM23 and AbTM45 IgG recognized CCR2B stably expressed in HEK 293 cells but did not react with mock-transfected cells (Fig. 1). Next we tested if the antisera can interfere with ligand binding indicating that these receptor domains are involved in the recognition of MCP-1. The transfected HEK 293 cells expressed on average 6.3 ± 0.6 fmol of CCR2B/10⁶ cells, which was determined by 125I-MCP-1 binding analysis. Incubation of the cells with preimmune IgG affected MCP-1 binding only insignificantly. In contrast, AbTM23 or AbTM45 IgG inhibited ligand binding substantially by about 80% when used individually, and by about 80% when used in combination (Fig. 2).

The results from the binding studies indicated that the first and second extracellular loops might be important for MCP-1 binding and receptor function. Therefore the effect of the antisera on MCP-1-induced chemotaxis of THP-1 monocytes was examined. As shown in Fig. 3, AbTM23 or AbTM45 IgG reduced the chemotactic response by about 50–70% compared with preimmune IgG or no antibody control. The combination of AbTM23 and AbTM45 IgG appeared to reduce chemotaxis even further, but the difference was statistically not significant and reflected the incomplete inhibition of MCP-1 binding by the antisera.

Identification of First Extracellular Loop as a Key Element for Receptor Function—To determine more directly that the first extracellular loop is involved in ligand binding and receptor function, we constructed a chimeric CCR2B. The first extracellular loop and the adjacent transmembrane domains between the restriction sites BalI and ClaI were exchanged with the corresponding region taken from CCR1 (Fig. 4A). Although the amino acid sequence of the first extracellular loop of these two receptors is 50% identical, CCR1 does not bind MCP-1. The chimeric receptor CH1 was stably expressed in HEK 293 cells, and ligand binding analysis was performed. Replacement of the

FIG. 1. Specific recognition of CCR2B by the antisera AbTM23 and AbTM45. Two antisera were made against the first (AbTM23) and second (AbTM45) extracellular loops of CCR2, and IgG fractions were prepared. To test their specificity, CCR2B-transfected HEK 293 cells (bold line) and mock-transfected controls (fine line) were incubated with either AbTM23 IgG (A) or AbTM45 IgG (B), followed by a fluorescein-labeled secondary antibody and analyzed by flow cytometry as described under “Experimental Procedures.”

FIG. 2. Inhibition of MCP-1 binding to CCR2B by the antisera AbTM23 and AbTM45. Transfected HEK 293 cells expressing CCR2B were incubated with 125I-MCP-1 and various concentrations of unlabeled ligand for 60 min at room temperature. Ligand binding analysis was performed as described under “Experimental Procedures” in the absence of antibody (□) or in the presence of preimmune IgG (■), AbTM23 IgG (●), AbTM45 IgG (▲), and a combination of both (▲). The cells were preincubated for 30 min at 4 °C with the IgG fractions at a final concentration of 10 µg/ml, after which radiolabeled ligand was added. Shown in the inset is the specific inhibition, which was determined after subtraction of nonspecific binding assessed in the presence of 30 µM unlabeled ligand from the total binding. In the absence of antibody, the transfected HEK 293 cells bound on average 6.3 ± 0.6 fmol MCP-1/10⁶ cells which was taken as 100%. No, MCP-1 binding in the absence of antibody; pre, 23, 45, and 23 + 45, MCP-1 binding in the presence of preimmune IgG, AbTM23 IgG, AbTM45 IgG and AbTM23/AbTM45 IgG, respectively. Data represent mean ± S.D. of three experiments.
first extracellular loop had a profound effect on the binding affinity (Fig. 4B) and increased the dissociation constant by about 10-fold compared with that of wild type CCR2B (Table I).

**Identification of Amino Acid Residues of the First Extracellular Loop that Are Important for Receptor Function**—To identify the individual amino acid residues that are necessary for receptor function, we compared the sequences of the wild type CCR2B and the chimeric receptor. We found several non-conservative differences in the amino acid sequence of the first extracellular loop that may be responsible for the diminished function associated with the chimeric receptor. They were located predominantly in the amino-terminal half of the loop proximal to the second transmembrane domain (Table I). This information was the basis for the selection of target amino acid residues, and their relative importance for receptor function was determined by site-directed mutagenesis and functional characterization of the mutant receptors expressed in HEK 293 cells. Three mutant receptors were generated in which amino acid residues of the wild type receptor were replaced by those of the chimeric receptor in clusters of two to three residues at a time. Substitution of the residues Ala\(^{39}\)-His\(^{102}\) with Ile-Asp-Tyr in MU1 or Ser\(^{101}\)-Ala\(^{102}\)-Ala\(^{103}\) with Lys-Leu-Lys in MU2 had no effect on the binding affinity, and the resulting dissociation constants were similar to that of the wild type receptor (Fig. 5). In contrast, changing the residues Asn\(^{104}\)-Lys\(^{106}\) to Asp-Asp, as in MU3, increased the dissociation constant significantly by about 10-fold (Table I).

Next we examined the ability of the mutant receptors to stimulate agonist-induced transmembrane signaling. MCP-1 induced a dose-dependent mobilization of intracellular calcium in transfected HEK 293 cells expressing the wild type CCR2B. In contrast, the chimeric receptor CH1 did not respond to MCP-1, and little change in \([Ca^{2+}]_i\), was observed, even at MCP-1 concentrations well in excess of that of the dissociation constant (Fig. 6). Although the mutations in MU1 and MU2 did not affect the ligand binding affinity, they affected transmembrane signaling. These mutant receptors were less efficient than the wild type CCR2 to mediate translocation of \([Ca^{2+}]_i\), and they required higher MCP-1 concentrations for the induction of a signaling event. Although the mutant MU3 displayed impaired ligand binding activity, it showed intact transmembrane signaling when stimulated with MCP-1 at concentrations above that of the dissociation constant, and the magnitude of the calcium response was higher than that seen with MU1 or MU2 (Fig. 6). These results suggested that amino acid residues of CCR2B that were mutated in MU1 and MU2 are important for transmembrane signaling, although they are not directly involved in MCP-1 binding.

**Activation of G Protein by the Mutant Receptors**—Receptor-mediated activation of G proteins requires both binding of agonist to the receptor and optimal interaction between receptor and G protein. If the necessary conformational rearrangement of the receptor protein is restricted, the productive coupling to G protein may be limited and may affect transmembrane signaling. To determine the relative efficacies with which wild type CCR2B and the mutant receptors activate G protein, plasma membranes were prepared from cells stably expressing the receptors, and the binding affinity of \(^{[35]}\text{S}GTP^S\) to G protein was determined. MCP-1 (30 nM) stimulated the binding of \(^{[35]}\text{S}GTP^S\) to membranes prepared from cells expressing CCR2B (Fig. 7A). Agonist-occupied chimeric receptor CH1 also stimulated binding of \(^{[35]}\text{S}GTP^S\) but with a lower affinity (Fig. 7A). The stimulation of G protein was specific for CCR2B because AbTM7C IgG, which is directed against the intracellular carboxyl-tail of CCR2B, largely blocked the MCP-1-induced binding of GTP-\(\gamma\)-S to G protein in the CCR2B (and mutant receptor)-transfected cells (Fig. 7, A and B). The binding affinities for \(^{[35]}\text{S}GTP^S\) stimulated by CCR2B and CH1 were 1.87 ± 0.35 and 7.58 ± 0.93 nM, respectively (Fig. 7, C and D, Table II).

The mutations in MU1 and MU2 did not affect the ligand binding affinities (Table I); however, they greatly decreased the efficiencies for receptor-mediated G protein stimulation (Fig. 8 and Table II). Both mutant receptors stimulated only a low affinity GTP-\(\gamma\)-S binding to G protein that was comparable to that mediated by the chimeric receptor CH1 (Fig. 8, A and B and Table II). In contrast, the mutant receptor MU3 stimulated GTP binding as efficiently as the wild type CCR2B (Fig. 8C and Table II). Evidently, the mutation in MU3 did not prevent any ligand-induced conformational changes that are critical for functional interaction between receptor and G protein. These observations indicate that the first extracellular loop may play an important role in both ligand binding and transmembrane signaling.

**DISCUSSION**

Chemokines play a fundamental role in host defense mechanisms. They induce a diverse array of biological responses in leucocytes through a distinct, structurally related family of seven-transmembrane domain G protein-coupled receptors. The functional interaction of chemokines with their specific receptors is generally very complex and may involve several distinct receptor segments (1, 33). A similar structural concept, in which the amino-terminal tail appears to play an essential role in the selective recognition of MCP-1, was also proposed for CCR2B (21). Additional evidence suggested that other extracellular receptor segments including the 3rd extracellular domain might be necessary for high affinity MCP-1 binding and possibly transmembrane signaling (34). Our results presented in this report support a multidomain binding model and demonstrate a critical role of the first extracellular loop in receptor function.
A CCR2-specific antiserum that was made against the first extracellular loop inhibited MCP-1 binding significantly by about 60%, which clearly indicated that this receptor segment is important for agonist recognition. A similar degree of inhibition was also found with the antiserum against the second extracellular loop, suggesting an equivalent contribution to agonist binding. In some cases antibodies may indirectly prevent receptor function by blocking ligand access through steric hindrance. To confirm that our antibodies prevented ligand binding by specifically masking the ligand-binding domain rather than indirectly through steric hindrance, we constructed a chimeric CCR2B. Of particular interest was the first extracellular loop because we found a dual function associated with this segment that we wanted to explore further. The results from the ligand binding studies clearly supported a critical role for this receptor segment. The substitution of this 15-residue domain including portions of the adjacent transmembrane helices with the corresponding sequence from CCR1 resulted in a 10-fold decrease in the ligand binding affinity.

### Table I

| Receptor | Sequence of 1st loop | $K_d$ (nM) | $B_{max}$ (fmol/10^6 cells) | EC50 (nM) |
|----------|---------------------|------------|-----------------------------|-----------|
| CCR2B    | ^99^AHSAANE^WF^G^NAM^CK114 | 0.44 ± 0.06 | 6.3 ± 0.6 | 24.7 ± 4.1 |
| CH1      | ^99^IDYKLKDD^WF^G^D^G^M^CK115 | 4.70 ± 0.80 | 6.2 ± 0.5 | >100 |
| MU1      | ^99^IDYS^A^A^N^E^W^F^G^N^A^M^CK115 | 0.20 ± 0.03 | 3.5 ± 0.4 | >100 |
| MU2      | ^99^AHKLKNEW^V^G^N^A^M^CK114 | 0.24 ± 0.09 | 3.9 ± 0.2 | >100 |
| MU3      | ^99^AHSA^D^D^D^D^W^V^F^G^N^A^M^CK114 | 4.66 ± 0.90 | 4.0 ± 0.7 | >100 |

### Fig. 4

**A**, schematic representation of the chimeric receptor CH1. The first extracellular loop of CCR2B (open cylinders) was replaced with the corresponding segment from CCR1 (shaded cylinders). **B**, ligand binding analysis. Transfected HEK 293 cells expressing wild type CCR2B (▲) or CH1 (●) were incubated with 125I-MCP-1, and various concentrations of unlabeled ligand and binding analyses were performed as described under “Experimental Procedures.” Background binding was determined on mock-transfected controls (■). Data represent mean ± S.D. of three experiments. Binding parameters were calculated with the LIGAND program and are given in Table I.

### Fig. 5

**A**, ligand binding analysis of mutant CCR2B. Mutant receptors were generated and the changes in their primary sequence are shown in Table I. Transfected HEK 293 cells expressing MU1 (■), MU2 (○), and MU3 (▲) were incubated with 125I-MCP-1, and binding analyses were performed as described in Fig. 4. Background binding was determined on mock-transfected controls (❖). Data represent mean ± S.D. of three experiments. Binding parameters were calculated with the LIGAND program and are summarized in Table I.

### Fig. 6

**A**, agonist-dependent increase of intracellular calcium in cells expressing wild type and mutant receptors. Transfected HEK 293 cells expressing wild type and the various mutant receptors were loaded with Indo-1 AM. Receptor-mediated change in $[Ca^{2+}]_i$ after stimulation with the indicated concentrations of MCP-1 was measured as the fluorescence ratio $F_{400\text{nm}}/F_{490\text{nm}}$ with the excitation set at 340 nm. Data are expressed as percent change of the fluorescence ratio relative to that achieved with wild type CCR2B after stimulation with a saturating dose of MCP-1 (300 nM), which was set at 100%.
Data are expressed as percent binding relative to no antibody control. The IgG fraction of the neutralizing anti-CCR2B antiserum AbTM7C (10 μg/ml) was included in the incubation mixture which contained the membranes from the CCR2B-transfected cells. D, extent of inhibition of [35S]GTPyS binding by AbTM7C. To determine the specific inhibition of maximal [35S]GTPyS binding by the antibody, membranes from CCR2B-transfected cells were incubated with 15 nM [35S]GTPyS in the presence of 10 μg/ml AbTM7C or 10 μg/ml preimmune IgG (pre Ab) and stimulated with 30 nM MCP-1. Data are expressed as percent binding relative to no antibody control (no Ab), which was taken as 100%. C, Scatchard plot of the specific [35S]GTPyS binding data obtained from membranes of CCR2B-expressing cells. D, Scatchard plot of the specific [35S]GTPyS binding data obtained from membranes of CH1-expressing cells. The average values of the binding parameters are summarized in Table II. Data are expressed as mean ± S.D. of three experiments.

properties of the chimeric receptor in transfected HEK 293 cells were also greatly impaired. Little transmembrane signaling was detectable even after stimulation of the chimeric receptor with saturating concentrations of MCP-1. These results suggested that the first extracellular loop of CCR2B was not only required for high affinity agonist binding but was also importantly involved in receptor activation.

This hypothesis was consistent with our results obtained on a set of mutant receptors that were constructed to dissect the functional properties of this loop in greater detail. The difference in the amino acid sequence of the first extracellular loop of CCR2B and its inhibition by the neutralizing anti-CCR2B antiserum AbTM7C. A, [35S]GTPyS binding experiments were carried out on membranes of transfected HEK 293 cells expressing wild type CCR2B (Δ) or the chimeric receptor CH1 (■) after stimulation with 30 nM MCP-1. Shown is the specific MCP-1-dependent binding of [35S]GTPyS to G protein determined as described under “Experimental Procedures.” In separate experiments, the specificity of the CCR2B-mediated stimulation was established. The IgG fraction of the neutralizing anti-CCR2B antiserum AbTM7C (10 μg/ml) was included in the incubation mixture which contained the membranes from the CCR2B-transfected cells (▲). B, extent of inhibition of [35S]GTPyS binding by AbTM7C. To determine the specific inhibition of maximal [35S]GTPyS binding by the antibody, membranes from CCR2B-transfected cells were incubated with 15 nM [35S]GTPyS in the presence of 10 μg/ml AbTM7C (AbTM7C) or 10 μg/ml preimmune IgG (pre Ab) and stimulated with 30 nM MCP-1. Data are expressed as percent binding relative to no antibody control (no Ab), which was taken as 100%. C, Scatchard plot of the specific [35S]GTPyS binding data obtained from membranes of CCR2B-expressing cells. D, Scatchard plot of the specific [35S]GTPyS binding data obtained from membranes of CH1-expressing cells. The average values of the binding parameters are summarized in Table II. Data are expressed as mean ± S.D. of three experiments.

transmembrane signaling of agonist-activated receptor. Although the ligand binding affinity was lower compared with CCR2B, the mutant receptor MU3 very efficiently activated G protein, which was reflected by its high affinity for GTPyS. The mutant receptor also induced a change of [Ca²⁺], in the transfected HEK 293 cells, although, as expected, at higher MCP-1 concentrations to compensate for the lower binding affinity. In contrast, the poor calcium response mediated by MU1 and MU2 cannot be explained by a low ligand binding affinity but was most likely caused by their very ineffective activation of G protein. The replacement of a positively charged residue (His³⁰⁵ → Tyr) together with the introduction of a negative charge (Asp³⁰⁹) in MU1, or the introduction of two positively charged residues in MU2 (Ser³¹⁰–Ala³¹²–Ala³¹³ → Lys–Leu–Lys) did not significantly affect the binding affinity but greatly reduced the efficacy of transmembrane signaling. These results suggest that the first extracellular loop contains microdomains with distinct properties that are necessary for optimal receptor function.

It should be noted that any exchange of amino acid residues could potentially perturb the local conformation and thereby indirectly affect receptor function. Although some of the mutations may change the physical properties within the microenvironment, unwanted conformational changes are probably not the cause for the specific effects of the mutations on transmembrane signaling or ligand binding, because they would more globally affect receptor function and presumably disrupt both to the same degree. The mutations in MU1 and MU2, however, very specifically affected only transmembrane signaling without changes in the ligand binding affinity, whereas MU3 showed impaired ligand binding but intact transmembrane signaling. Taken together these observations strongly suggest a direct role of the mutated amino acid residues in either signal transduction or ligand binding.

Our finding that ligand binding can be dissociated from receptor activation is consistent with a hypothetical model that predicts a two-step mechanism for CCR2 activation (32). In this model, MCP-1 binds first to the amino-terminal tail before it interacts in the second step with other extracellular domains to initiate signal transduction. Multiple activation steps were also proposed for other chemotaxtant receptors. A single point mutation in the second transmembrane domain of the N-formyl peptide receptor was shown to inhibit the signaling pathway without changing the ligand binding affinity, demonstrating that distinct amino acid residues are involved in these activation steps (35).

These results were rather unexpected since extracellular loops of G protein-coupled receptors are generally not believed to be associated with signaling events. Because G proteins are located on the cytoplasmic face of the plasma membrane, it seems logical that intracellular hydrophilic receptor segments would form the most likely sites of interaction with a G protein.
Results from a limited study suggested the carboxyl-terminal tail and third intracellular loop of CCR2B as candidate sites for the selective G protein coupling (12, 36). This model is in agreement with our finding that the antisera AbTM7C, which is directed against the intracellular carboxyl-terminal tail of CCR2B, blocked quite effectively the MCP-1-induced activation of G protein. In the free form, the receptor is thought to exist in an inactive conformation, ineffectual for G protein activation. The binding of agonist then induces the change of the receptor conformation that is required for functional coupling to G protein. Although the exact molecular mechanisms are still unknown, our data suggest a model in which the interaction of MCP-1 with specific amino acid residues of the first extracellular loop is central for the activation of CCR2B and subsequent transmembrane signaling. Charged amino acid residues appear functionally critical, and their replacement may prevent the effective change of the receptor conformation.

In summary, our studies show that the functional interaction of MCP-1 with CCR2 involves multiple receptor domains including the first extracellular loop. Our data further suggest that this receptor segment contains two distinct microdomains, one that supports high affinity ligand binding and one that is central to receptor activation. The critical components for ligand binding and receptor activation lie between amino acids 100 and 105, although other regulatory segments may be involved. In addition to providing insights into the mechanisms of receptor activation, the identification of an extracellular regulatory sequence distinct from the ligand-binding sites may offer a new pharmacological approach to the synthesis of non-peptide antagonists for therapeutic use.

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