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# The chemokine monocyte chemoattractant protein-1 induces functional responses through dimerization of its receptor CCR2

**(chemotaxis/inflammation/HIV type 1)**

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**ABSTRACT** **Cytokines interact with hematopoietin superfamily receptors and stimulate receptor dimerization. We demonstrate that chemoattractant cytokines (chemokines) also trigger biological responses through receptor dimerization. Functional responses are induced after pairwise crosslinking of chemokine receptors by bivalent agonistic antichemokine receptor mAb, but not by their Fab fragments. Monocyte chemoattractant protein (MCP)-1-triggered receptor dimerization was studied in human embryonic kidney (HEK)-293 cells cotransfected with genes coding for the CCR2b receptor tagged with YSK or Myc sequences. After MCP-1 stimulation, immunoprecipitation with Myc-specific antibodies revealed YSK-tagged receptors in immunoblotting. Receptor dimerization also was validated by chemical crosslinking in both HEK-293 cells and the human monocytic cell line Mono Mac 1. Finally, we constructed a loss-of-function CCR2bY139F mutant that acted as a dominant negative, blocking signaling through the CCR2 wild-type receptor. This study provides functional support for a model in which the MCP-1 receptor is activated by ligand-induced homodimerization, allowing discussion of the similarities between bacterial and leukocyte chemotaxis.**

The chemokines are a group of cytokines that promote leukocyte recruitment to inflammatory tissues, stimulate leukocyte exocytosis, and induce hematopoiesis (1, 2). Based on the relative position of the two first cysteine residues, chemokines are classified in four groups, termed $\alpha$, $\beta$, $\gamma$, and $\delta$ or CC, CXC, C, and $CX_3C$. In mammals, chemokine target cells are found in a large variety of organs, including immunological, respiratory, hematopoietic, reproductive, and secretory tissues. This family of chemoattractant proteins has been implicated in tumor rejection, as well as in numerous chronic malignancies, including immunological disease, fibrosis, glomerulonephritis (3, 4), and HIV type 1 infection (5).

The chemokine receptors belong to the family of single-chain, seven-transmembrane spanning receptors coupled to G proteins (6). Based on sequence homology, two major chemokine receptors have been described, the CCR and the CXCR receptors, which interact with the CC and CXC chemokines, respectively (1). To date, 10 CC and five CXC receptors have been reported, as have several putative receptors whose ligands remain to be elucidated. The extracellular loops of these receptors act in concert to bind the chemokine ligand, whereas different intracellular regions are involved in transducing the chemokine signal. Chemokine receptors generally are coupled to $G_{\alpha i}$ proteins (7), rendering cellular responses sensitive to pertussis toxin (PTX). There are nonetheless several examples of chemokine receptors that couple to both PTX-insensitive or PTX-sensitive G proteins, as is the case of the C5a receptor (8). Ligand binding triggers a number of biochemical pathways that lead to changes in intracellular cAMP levels (9), phospholipase activation (10), increases in tyrosine phosphorylation (11, 12), increased association of Src family kinases with $p21^{ras}$ (13), and mitogen-activated protein kinase cascade activation (12, 14). We recently have shown that chemokine signaling requires Janus family kinases (JAK) kinases (15). Monocyte chemoattractant protein (MCP)-1 triggers rapid tyrosine phosphorylation of the CCR2, in which the tyrosine at position 139 plays a critical role, as well as the activation of the JAK2/STAT3 pathway in a PTX-independent manner. In fact, replacement of Y139 by phenylalanine renders a loss-of-function CCR2b mutant (15). It also has been demonstrated that regulated on activation, normal T cell expressed and secreted (RANTES) and macrophage inflammatory protein-1$\alpha$ trigger protein tyrosine kinases and STAT1 and STAT3 activation (16), and that a *Dictyostelium* signal transducers and activators of transcription (STAT) protein is activated through the chemoattractant cAR1 serpentine receptor in a G protein-independent manner (17).

By using a variety of approaches, we show here that MCP-1 induces functional responses through CCR2 receptor dimerization. First, monovalent Fab fragments obtained from an agonist anti-CCR2 mAb (CCR2–02) (18, 19) do not promote functional responses ($Ca^{2+}$ mobilization, cell migration, or JAK/STAT pathway activation) as the bivalent antibody does. This function is restored when the Fab is crosslinked by anti-Fab antibodies, indicating that a complex of at least two receptors is required to induce a functional response. Second, by using cells cotransfected with CCR2b receptor cDNA tagged in the N-terminal extracellular domain with Myc or YSKFDT (YSK) coding sequences, we demonstrate in Myc-derived immunoprecipitates that YSK-tagged CCR2b receptors were observed only after MCP-1 activation, indicating that the natural ligand induces receptor oligomerization. Third, we demonstrate that the previously described CCR2bY139F mutant form of CCR2b (15), which dimerizes after MCP-1 stimulation but does not trigger any signaling pathway, acts as a CCR2b dominant negative mutant, blocking chemokine responses through its ability to form nonproductive complexes with partners containing the functional tyrosine domain.

These observations, together with other reports describing the dimerization of G protein-coupled receptors (GPCRs) (20), led us to postulate a mechanism for ligand-induced

Abbreviations: MCP, monocyte chemoattractant protein; HEK, human embryonic kidney; YSK, YSKFDT peptide; PTX, pertussis toxin; JAK, Janus family kinases; GPCR, G protein-coupled receptor; STAT, signal transducers and activators of transcription; SDF-1$\alpha$, stromal cell-derived factor 1$\alpha$; RANTES, regulated on activation, normal T cell expressed and secreted.

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activation, in which the chemokine receptors undergo oligomerization in response to chemokines.

## MATERIALS AND METHODS

**Biological Materials.** Mono Mac 1 (DSM ACC252) and human embryonic kidney (HEK)-293 cells (ATCC TIB202) were from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and the American Type Culture Collection, respectively. HEK-EBNA293 cells were obtained from Invitrogen. Antibodies used include the anti-CCR2 mAb CCR2–02, CCR2–03, CCR2–05, and anti-YSK generated in our laboratory (18, 19), anti-Myc (clone 9E10; Santa Cruz Biotechnology), rabbit anti-JAK2 (Santa Cruz Biotechnology), and rabbit anti-PTyr antibody (Promega). MCP-1, RANTES, and stromal cell-derived factor 1$\alpha$ (SDF-1$\alpha$) were from PeproTech (London).

**Production of Fab Fragments.** Fab fragments were obtained by pepsin digestion of purified CCR2–02 and isotype-matched mAbs. $F(ab')_2$ fragments were separated, reduced, and alkylated, and the resulting monovalent Fab fragments were dialyzed against PBS. Rabbit anti-mouse IgG Fab antibodies were produced and purified as described (21).

**Construction of cDNA Expression Vectors.** CCR2b and CCR5 cDNA was cloned in the *Kpn*I–*Xba*I sites of pcDNA3 (Invitrogen). Two *Kpn*I–*Cla*I CCR2b fragments were PCR-amplified by using 5′ oligonucleotides to introduce the sequences coding for the tags Myc (TCGGTACCATGGAGCAGAAGCTGATCTCCGAGGAAGACCTGTCCACATCTTCGTTCTCG) or YSK (TCGGTACCATGTATAGTAAGTTCGATACTCTGTCCACATCTCGTTCTCG) and 3′ oligonucleotide (ATCTTATCGATTGTCAGGAGG). These fragments, as well as CCR2b-pCDNA3, were double-digested with *Kpn*I–*Cla*I, gel purified, and ligated, and the CCR2b-pcDNA3 *Kpn*I–*Cla*I fragment was replaced with Myc- or YSK-coding PCR fragments; both were confirmed by DNA sequencing. CCR2bY139F was constructed as described (15). YSK-tagged CCR2bY139F was obtained by using the above strategy.

**Transfection.** HEK-293 cells were cotransfected with Myc-CCR2b and YSK-CCR2b constructions by calcium phosphate precipitation. Transfected cells were selected in G-418 (Calbiochem) and analyzed by flow cytometry for receptor expression by using anti-CCR2b, anti-Myc, and anti-YSK antibodies. For transient transfection experiments, HEK-EBNA 293 cells were incubated with Lipofectamine (GIBCO/BRL) and DNA (22).

**Flow Cytometry Analysis.** Briefly, cells were plated ($2.5 \times 10^5$ cells/well) and incubated with 50 $\mu$l/well of biotin-labeled mAb (5 $\mu$g/ml, 30 min, 4°C) (18). After washing, fluorescein isothiocyanate-labeled streptavidin (Southern Biotechnology Associates) was added and incubated, and cell-bound fluorescence was measured at 525 nm in a Profile XL flow cytometer (Coulter). For internalization studies, CCR2b- or CCR2bY139F-transfected HEK-293 cells were untreated or preincubated with 20 nM MCP-1 ($10 \times 10^6$ cells/ml, 20 min, 37°C), and receptor levels were analyzed.

**Calcium Determination.** Intracellular calcium concentration changes were monitored by using the fluorescent probe Fluo-3,AM (Calbiochem), as described (19). Briefly, cells ($2.5 \times 10^6$ cells/ml), untreated or treated for 16 h with PTX (0.1 $\mu$g/ml), were incubated with 10 $\mu$l/$10^6$ cells of Fluo-3,AM (300 $\mu$M in DMSO) for 15 min at 37°C. Cells then were washed, resuspended in complete medium containing 2 mM $CaCl_2$, and maintained at 4°C until just before stimulation. Calcium mobilization was measured at 37°C in an EPICS XL flow cytometer at 525 nm (Coulter) and included background stabilization, as well as determination of probe loading by using an ionophore (5 $\mu$g/ml ionomycin, Sigma).

**Cell Migration.** As described earlier (19), Mono Mac 1 or transfected HEK-293 cells were placed in the upper chamber of endothelial cell-coated transmigration chambers (5 or 8 $\mu$m, respectively; Costar), and increasing MCP-1, CCR2–02 mAb, or CCR2–02 Fab concentrations were added to the lower chamber. Plates were incubated (120 min, 37°C, 5% $CO_2$) and cell migration was expressed as a percentage of the maximum chemokine-induced response. To block MCP-1-induced chemotaxis, Mono Mac 1 cells ($10^6$/ml) were preincubated for 30 min at 4°C with Fab from CCR2–02 mAb (5 $\mu$g/ml) or from an isotype-matched control mAb (FabC, 5 $\mu$g/ml). Cells were allowed to migrate in response to 10 nM MCP-1 as before. Cell migration was expressed as a migration index and calculated as the *x*-fold increase in migration observed over the medium control.

**Immunoprecipitation, Crosslinking, SDS/PAGE, and Western Blot Analysis.** Serum-starved cells ($10^7$ cells/ml) were untreated or stimulated as indicated, and the reaction was terminated by adding 1 ml of cold PBS and centrifuging. Cells were lysed and supernatants were precleared as described (15). The supernatant then was incubated with the corresponding monoclonal CCR2–03 (5 $\mu$g/ml in PBS), anti-Myc (5 $\mu$g/ml), or polyclonal anti-PTyr antibody (1.5 $\mu$g/ml; overnight, 4°C), followed by incubation with anti-mouse or anti-rabbit IgG-agarose (20 $\mu$g/$10^7$ cells) for 60 min. Precipitates then were washed, resolved in SDS/polyacrylamide gels, and transferred to nitrocellulose membranes (15). After blocking with 10% nonfat dry milk in PBS, the membrane was incubated with the appropriate antibody (overnight, 4°C), followed by goat anti-mouse Ig-PO (Pierce) or goat anti-rabbit Ig-PO (Dako), and the reaction was developed by using ECL (Amersham Pharmacia).

For crosslinking, cells were treated with 10 $\mu$l of 100 mM disuccinimidyl suberate (Pierce) for 10 min at 4°C with continuous rocking and washed three times with 1 ml of PBS before lysis.

When stripping was required, membranes were treated as described (15), reprobed, and developed as above. Protein loading was controlled by using a protein detection kit (Pierce) and, when necessary, by reprobing membranes with the immunoprecipitating antibody.

## RESULTS AND DISCUSSION

**MCP-1 Agonist mAbs Induce CCR2 Receptor Dimerization.** The Mono Mac 1 monocyte cell line expresses the CCR2 receptor (23), as does the HEK-293 cell line transfected with the CCR2b receptor. We screened monoclonal anti-CCR2 receptor antibodies for MCP-1 agonist activity and found that the N-terminal domain-specific CCR2–02 antibody is as potent as MCP-1 in promoting $Ca^{2+}$ mobilization (Fig. 1 *A* and *B*) and cell transmigration. Both CCR2–02 and MCP-1 responses are blocked by treatment with PTX (Fig. 1 *D* and *E*) or tyrphostin B42 (a JAK2 tyrosine kinase inhibitor) (not shown). When Fab fragments prepared from the CCR2–02 antibody were tested in $Ca^{2+}$ mobilization assays (Fig. 1*G*), no response was triggered. CCR2–02 Fab and rabbit anti-mouse IgG Fab were added sequentially to Fluo-3,AM-loaded Mono Mac 1 cells to reconstitute bivalency; crosslinking of the CCR2–02 Fab in this manner reconstituted triggering of $Ca^{2+}$ mobilization in a PTX-sensitive manner (Fig. 1 *C* and *F*). No response was obtained when cells were stimulated with anti-Fab alone or with crosslinked irrelevant Fab used as control (Fig. 1 *H* and *I*). While $Ca^{2+}$ influx promoted by MCP-1 and CCR2–02 requires only their interaction with CCR2, the CCR2–02 Fab-induced $Ca^{2+}$ mobilization requires Fab binding to the CCR2 and interaction between this fragment and the antibody used as crosslinker, which could explain the differences observed in the kinetics.

When transmigration was studied by MCP-1 and CCR2–02 titration, we found that this activity is lost at high concentrations in both cases (Fig. 2*A*). This bell-shaped dose-response

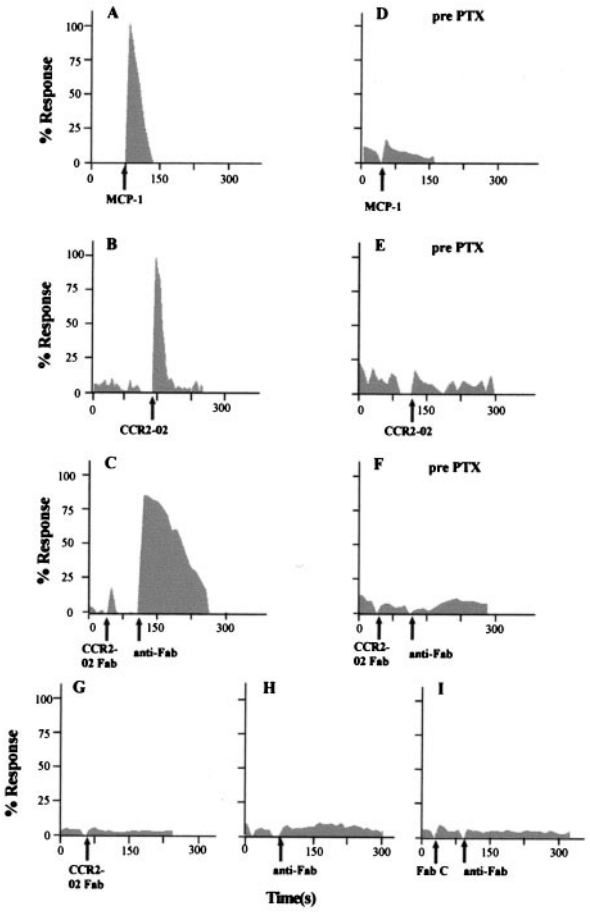


FIG. 1. $Ca^{2+}$ mobilization triggered by MCP-1, CCR2–02 mAb, or crosslinked CCR2–02 Fab. Induction of $Ca^{2+}$ mobilization was promoted by 10 nM MCP-1 (Peprotech) alone or in the presence of PTX (*A* and *D*, respectively), 15 nM CCR2–02 mAb (*B* and *E*), and 1 $\mu$g/ml of CCR2–02 Fab plus 50 $\mu$g/ml of rabbit anti-mouse IgG Fab (*C* and *F*) in Mono Mac 1 cells loaded with Fluo-3,AM, as described. Results are expressed as a percentage of the chemokine-induced calcium response. Shown are the results of one representative experiment of five performed. (*G-I*) $Ca^{2+}$ mobilization promoted by 1 $\mu$g/ml of CCR2–02 Fab alone, 50 $\mu$g/ml of rabbit anti-mouse IgG Fab alone, and 1 $\mu$g/ml of isotype-matched control Fab plus 50 $\mu$g/ml of rabbit anti-mouse IgG Fab. Fab and anti-Fab antibody were added sequentially; cells were washed between treatments. Arrows indicate stimulus addition.

effect has been described for other chemokines (24) and is not caused by a toxic effect of the chemokine or antibody used, as cell proliferation was unaffected by high concentrations of any of the ligands studied (data not shown). When the CCR2–02 Fab was tested on cell migration, no effect was observed (Fig. 2*A*). CCR2–02 Fab, but not control Fab, blocked the MCP-1 response (Fig. 2*B*). To rule out the possibility that the inability of the Fab to activate the CCR2 receptor is caused by changes in its binding capacity, we performed flow cytometry assays showing that both the CCR2–02 IgG and the corresponding Fab bind equally well to the CCR2 in several cell types (Mono Mac 1, peripheral blood lymphocytes, and CCR2b-transfected HEK-293 cells). Furthermore, both molecules block MCP-1-induced Mono Mac 1 cell migration at equimolecular concentrations (50% inhibition at 20 nM for both CCR-02 Fab and CCR2–02 IgG). Finally, equal binding curves were obtained for both molecules in enzyme immunoassays against CCR2(24–38) and CCR2(1–48) peptides.

These results are consistent with the requirement for at least two receptors for MCP-1-induced functional responses. To demonstrate this, we show that CCR2–02, but not CCR2–02

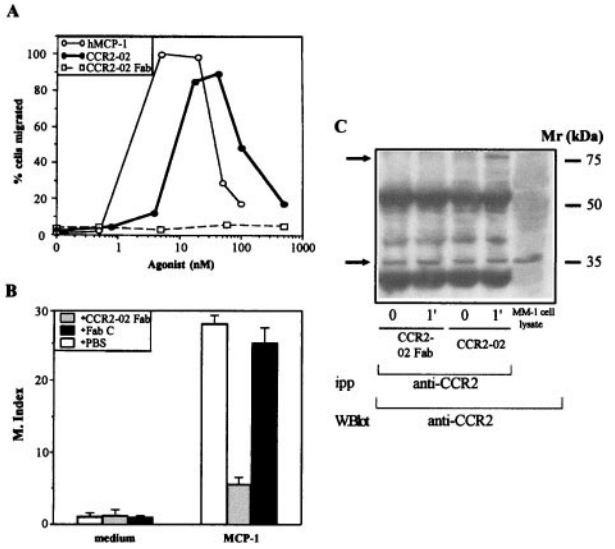


FIG. 2. The CCR2–02 agonist antibody triggers cell migration and CCR2 receptor dimerization. (*A*) Mono Mac 1 cells in the upper chamber of the transwell were incubated with increasing concentrations of MCP-1, CCR2–02, or CCR2–02 Fab in the lower chamber. Cells migration is expressed as a percentage of the maximum chemokine-induced response. (*B*) Chemotaxis was assessed as above, using Mono Mac 1 cells ($10^6$/ml) preincubated with 5 $\mu$g/ml of CCR2–02 Fab or 5 $\mu$g/ml of isotype-matched control mAb (FabC). After washing, cells were allowed to migrate in response to 10 nM MCP-1. The cell migration index (MI) was calculated and expressed as in *Materials and Methods*. Data represent the mean ± SD of triplicate determinations. (*C*) Mono Mac 1 cells were stimulated for 1 min with 15 nM CCR2–02 mAb or 1 $\mu$g/ml of CCR2–02 Fab. After crosslinking, cell lysates were immunoprecipitated by using anti-CCR2 Ab and analyzed in Western blot with anti-CCR2 Ab. As a positive control an unstimulated, unimmunoprecipitated Mono Mac 1 cell lysate was immunoblotted with anti-CCR2 mAb. Arrows indicate the receptor monomer (38 kDa) and dimer (75 kDa).

Fab fragments, trigger CCR2 dimerization. Disuccinimidyl suberate-mediated crosslinking in Mono Mac 1 cells was carried out after CCR2–02 mAb or Fab stimulation. We observed a high molecular mass receptor species (75 kDa) after stimulation with intact mAb, but not with the corresponding Fab fragment (Fig. 2*C*). This finding corresponds to the expected molecular mass of two CCR2 molecules, as assessed by immunoprecipitation with anti-CCR2 antibodies and Western blot developed with anti-CCR2 antibodies; CCR2 does not appear in CCR2–02-stimulated, crosslinked cell lysates immunoprecipitated with an isotype-matched control antibody (not shown).

These results show that dimerization of the CCR2b chemokine receptor is required to activate receptor-associated signal transduction pathways. They also indicate that antibodies specific for the transmembrane-spanning receptor protein can effectively initiate a signaling cascade leading to cell migration and to $Ca^{2+}$ influx. Studies performed in the 1980s showed that antiidiotypic antibodies to anti-$\beta$-adrenergic ligands can induce biological effects (25); our results provide additional information on the ability of mAb to trigger seven-transmembrane G protein-associated receptors.

**The MCP-1 Chemokine Induces CCR2 Receptor Dimerization.** To determine whether dimerization is the mechanism through which the CCR2 receptor is activated by its natural ligand, MCP-1, we constructed two expression vectors carrying CCR2b receptor cDNA-tagged in the N-terminal extracellular domain with Myc or YSK coding sequences (Fig. 3*A*), which subsequently were transfected into HEK-293 cells. The transfected constructs were expressed on the cell surface, as detected in flow cytometry using specific antibodies and mock-

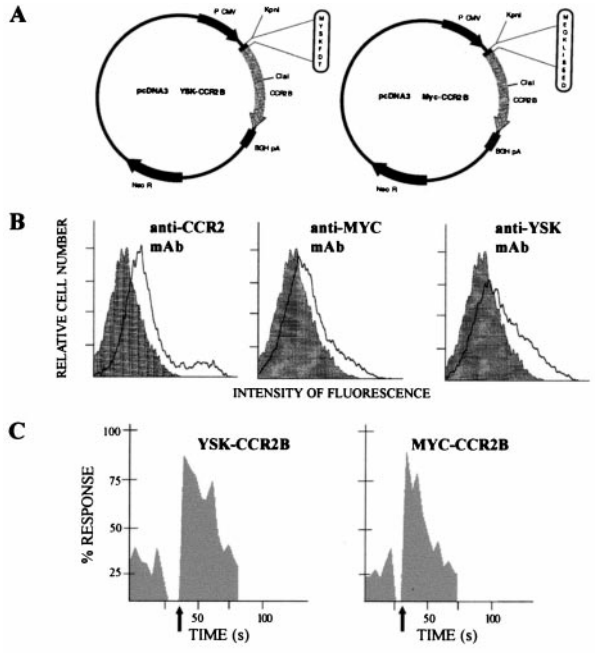


FIG. 3. The tagged CCR2b constructs are expressed on cell surface and retain MCP-1 receptor activity. (*A*) HEK-293 cells were transfected with the indicated expression construct for Myc-CCR2b or YSK-CCR2b. (*B*) Stably cotransfected HEK-293 cells were stained with the appropriate mAb to demonstrate membrane expression of CCR2b (*Left*), Myc-CCR2b (*Middle*), and YSK-CCR2b (*Right*). Mock-transfected HEK-293 cells (shaded area) were used as a control for each antibody. Shown is a representative experiment of five performed. (*C*) $Ca^{2+}$ mobilization was determined as in Fig. 1, after stimulation with 10 nM MCP-1 in transiently transfected HEK-EBNA 293 cells bearing Myc-CCR2b or YSK-CCR2b constructs. Arrows indicate stimulus addition.

transfected cells as control (Fig. 3*B*). Tag-CCR2b fusion proteins are present in anti-Myc or anti-YSK immunoprecipitates of cell lysates developed with anti-CCR2 antibodies in Western blot (not shown). Both constructions are functional, as measured by $Ca^{2+}$ mobilization in response to MCP-1, not observed in mock-transfected cells (Fig. 3*C*). To verify CCR2 receptor dimerization after MCP-1 binding, anti-Myc immunoprecipitates of cell lysates were developed in Western blot with anti-YSK antibodies. In Myc-derived immunoprecipitates, YSK-tagged CCR2b receptors (38 kDa) were observed only after MCP-1 activation, but not in the absence of activation, demonstrating that MCP-1 indeed triggers CCR2 receptor dimerization (Fig. 4*A*, *Left*). The membrane was stripped and reprobed with anti-CCR2 antibody; no differences were observed in the amount of CCR2 in each lane (Fig. 4*A*, *Right*). No evidence was obtained for coprecipitation in MCP-1-stimulated cell lysates from single transfectants with the Myc or the YSK construct, even when immunoprecipitates from $4 \times 10^7$ cells were developed with the reciprocal antitag antibody, whereas coprecipitation was always readily observed in MCP-1-stimulated double transfectant cell lysates from $2 \times 10^6$ cells performed in parallel.

To validate the coimmunoprecipitation results and to compare them with those obtained in growth factor-induced dimerization of receptor tyrosine kinases (26), we performed crosslinking experiments in HEK-293 cells cotransfected with YSK-CCR2b and Myc-CCR2b constructs. After MCP-1 stimulation and disuccinimidyl suberate crosslinking, we detected the high molecular mass receptor species (75 kDa) corresponding to the expected molecular mass of two CCR2 molecules, as assessed by immunoprecipitation with anti-Myc antibodies and Western blot with anti-YSK antibodies (Fig. 4*B*, *Left*). This was

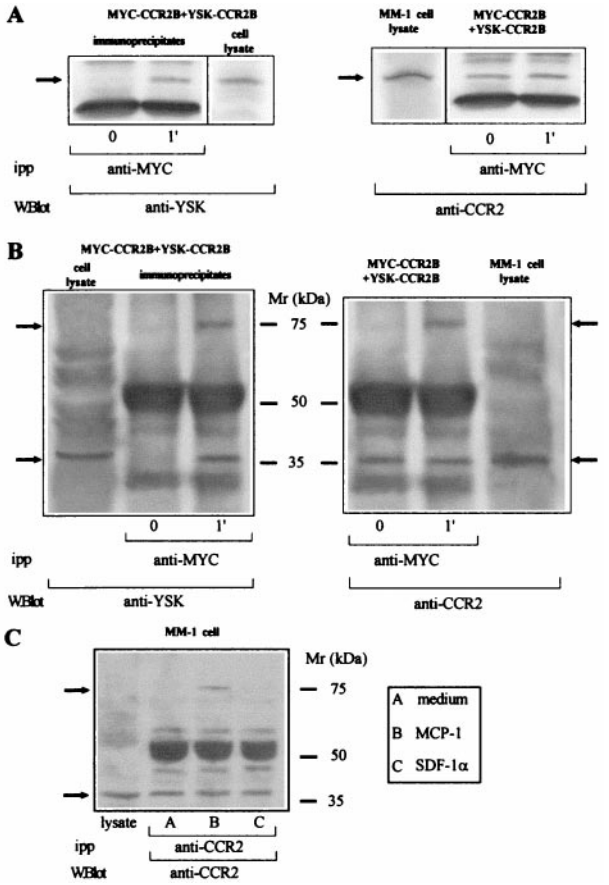


FIG. 4. MCP-1 induces CCR2 dimerization in mammalian cells. (*A*) HEK-293 cells cotransfected with the expression vector for Myc-CCR2b and YSK-CCR2b were stimulated for 1 min with 10 nM MCP-1. Cell lysates were immunoprecipitated with anti-Myc mAb and analyzed in Western blot with anti-YSK mAb; as a positive control, cotransfected HEK-293 cell lysates were immunoblotted with anti-YSK mAb (*Left*). The membrane was stripped and reblotted with anti-CCR2 mAb as a protein loading control; Mono Mac 1 cell lysates were immunoblotted with the same anti-CCR2 mAb as a control (*Right*). Arrows indicate the monomeric CCR2b (38 kDa). (*B*) Serum-starved HEK-293 cells cotransfected with the expression vector for Myc-CCR2b and YSK-CCR2b ($15 \times 10^6$) were unstimulated or stimulated for 1 min with 10 nM MCP-1 at 37°C. After crosslinking, cell lysates were immunoprecipitated by using anti-Myc antibody and analyzed in Western blot with anti-YSK. As a positive control, cotransfected HEK-293 cell lysates were immunoblotted with anti-YSK (*Left*). CCR2 loading was controlled as in *A* (*Right*). Arrows indicate the receptor monomer (38 kDa) and dimer (75 kDa). (*C*) Serum-starved Mono Mac 1 cells were untreated (lane A) or stimulated for 1 min with 10 nM MCP-1 (lane B) or 10 nM SDF-1α (lane C). Cells then were crosslinked, lysed, immunoprecipitated with anti-CCR2 (CCR2–03) mAb, and immunoblotted with anti-CCR2 (CCR2–05) mAb. Arrows indicate the receptor monomer (38 kDa) and dimer (75 kDa). As a positive control, a Mono Mac 1 cell lysate immunoblotted with anti-CCR2 (CCR2–05) mAb is shown.

confirmed by reprobing the blot with anti-CCR2 mAb (Fig. 4*B*, *Right*). To discard the possibility that these observations were the result of the use of a nonlymphoid cell line, we used untransfected Mono Mac 1 cells, which constitutively expresses functional CCR2 and CXCR4. After MCP-1 stimulation and disuccinimidyl suberate crosslinking, the same high molecular mass receptor species was detected, corresponding to the expected molecular mass of two CCR2 molecules (75 kDa), by immunoprecipitation and Western blot with anti-CCR2 antibodies (CCR2–03 and CCR2–05, respectively) (Fig. 4*C*). No high molecular mass CCR2 species was observed when cells were stimulated with SDF-1α, ruling out nonspecific interac-

tions caused by the assay conditions. We therefore conclude that receptor dimerization is a critical event linked to specific ligand activation of the chemokine receptor.

**CCR2 Dimerization Triggers JAK Kinase Recruitment for Activation of the Signaling Pathway.** Chemokine signaling is known to involve activation of the $G_i$ pathway, and we recently have described that $G_i$ associates rapidly to CCR2 after MCP-1 activation. As occurs in cytokine responses, MCP-1 promotes tyrosine phosphorylation of CCR2, a process involving activation of JAK2, which is rapidly associated to the receptor (15).

In accordance with this upstream role for JAK2 in chemokine signaling, we analyzed the consequences of treatment of quiescent Mono Mac 1 cells with MCP-1, CCR2–02, or CCR2–02 Fab. Exposure of cells to CCR2–02 or MCP-1, but not CCR2–02 Fab, induces JAK2 tyrosine phosphorylation. JAK2 phosphorylation also was produced, however, when bivalent anti-Fab IgG antibody was administered to cells after CCR2–02 Fab binding. Anti-Fab IgG alone was inactive (Fig. 5). As described (25), MCP-1 induces STAT3 phosphorylation and association to the CCR2 receptor, as does the bivalent CCR2–02 antibody. In contrast, CCR2–02 Fab failed to do so, although this property is restored after Fab crosslinking by anti-Fab IgG antibody, reinforcing our preliminary hypothesis that a functional response requires at least two CCR2 molecules (not shown).

We previously showed that MCP-1 induces $Ca^{2+}$ mobilization in HEK-293 cells expressing the CCR2b wild-type receptor, but not when they express the CCR2bY139F mutant or are mock-transfected. This result is because of the inability of the CCR2bY139F mutant to be phosphorylated on tyrosine, rendering it unable to recruit and trigger JAK2 phosphorylation, impeding $G_i$ association to this mutant receptor and, thus, activation of the signaling pathway (15). Furthermore, using transfected HEK-293 cells, we tested the MCP-1-induced internalization of both CCR2b and CCR2bY139F. Whereas CCR2b disappears from the cell surface after MCP-1 treatment (60% loss after 20 min), CCR2bY139F levels were unchanged, as demonstrated by flow cytometry analysis using anti-CCR2 antibodies (not shown). These data indicate once again that mutation of Y139 renders a completely nonfunctional receptor. Tyrosine 139 is located in the second intracellular loop and forms part of the DRY sequence, a highly conserved motif in chemokine receptors that is essential for agonist-mediated GPCR activation (27, 28). Wild-type and CCR2bY139F mutant receptor expression were identical in transfected HEK-293 cells, as measured by flow cytometry with specific antibodies (not shown). Coexpression of the wild-type CCR2b and the CCR2bY139F mutant in HEK-293 cells at a 1:2 ratio shows that the response to MCP-1 is impaired (Fig. 6*A*). As a control, we show that coexpression of CCR5 and CCR2bY139F do not affect the response to RANTES (Fig. 6*A*). Furthermore, MCP-1-triggered migration of CCR2b receptor-transfected HEK-293 cells is blocked when they are cotransfected with CCR2bY139F (Fig. 6*B*). We also have observed that the CCR2bY139F mutant undergoes receptor dimerization in response to MCP-1, although it is unable to trigger downstream signals (Fig. 6*C*, *Left*). Interestingly, these two receptors undergo heterodimerization after MCP-1 stimulation, impairing downstream responses to MCP-1 (Fig. 6*C*, *Right*). It is known that receptor clustering occurs during the initiation of internalization. The possibility that our observations were the result of this process is discarded, as the CCR2bY139F mutant dimerizes in response to MCP-1 but does not internalize. We therefore conclude that the CCR2bY139F acts as a CCR2b dominant negative mutant, which blocks chemokine responses by its ability to form nonproductive complexes with partners containing the functional domain, and demonstrates the biological significance of dimerization in chemokine responses.

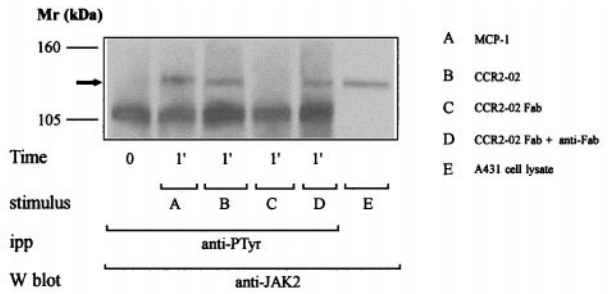


FIG. 5. MCP-1, CCR2–02, and crosslinked CCR2–02 Fab induce tyrosine phosphorylation of JAK2 kinase. Mono Mac 1 cells were stimulated for 1 min with 10 nM MCP-1, 15 nM CCR2–02 mAb, 1 $\mu$g/ml of CCR2–02 Fab plus 50 $\mu$g/ml of rabbit anti-mouse IgG Fab or 1 $\mu$g/ml of CCR2–02 Fab alone. Cell lysates were immunoprecipitated with anti-PTyr (PY20), electrophoresed, and transferred to nitrocellulose membranes. The Western blot was developed with an anti-JAK2 antibody. As a positive control, epidermal growth factor-stimulated A431 cell lysates were tested in Western blot with the same anti-JAK2 antibody. Arrow indicates the position of JAK2.

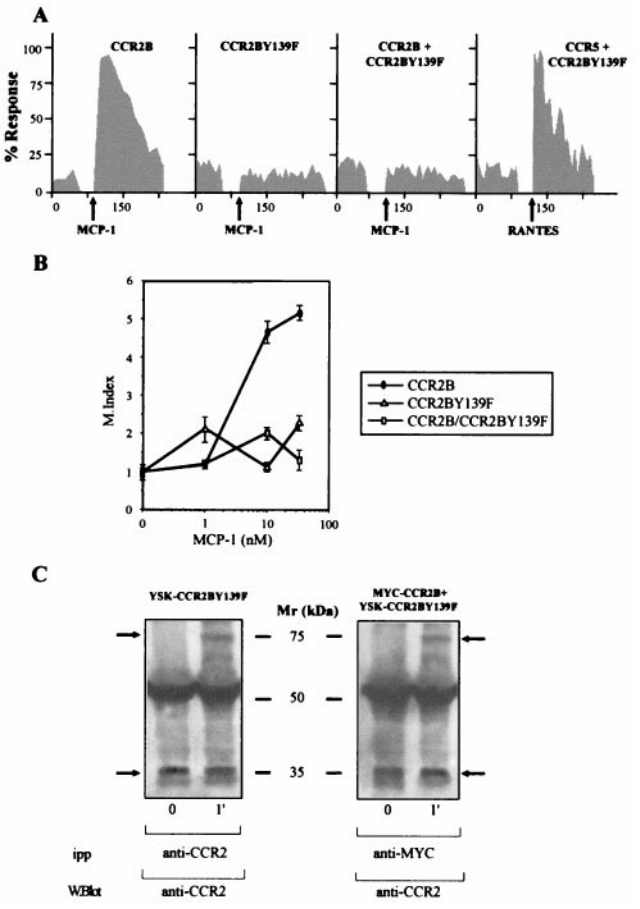


FIG. 6. CCR2bY139F loss-of-function mutant acts as a dominant negative mutant. (*A*) HEK-293 cells were transfected with CCR2b and CCR2bY139F alone, cotransfected with CCR2b and CCR2bY139F, or cotransfected with CCR5 and CCR2bY139F, as indicated. $Ca^{2+}$ mobilization in response to 10 nM MCP-1 or 10 nM RANTES was measured as in Fig. 1. Arrows indicate stimulus addition. (*B*) Migration of the indicated transfected cells in response to 10 nM MCP-1 was determined in 8 $\mu$m transmigration chambers, as in Fig. 2*B*. Data represent the mean of triplicate determinations ±SD. (*C*) HEK-293 cells transfected with YSK-tagged CCR2bY139F alone or cotransfected with Myc-CCR2b were stimulated with 10 nM MCP-1 for 1 min at 37°C, crosslinked, and analyzed as in Fig. 2*C*. Arrows indicate the receptor monomer (38 kDa) and the dimer (75 kDa).

Oligodimerization represents a subset of protein–protein interactions that generates considerable functional diversity (26). It frequently operates in signal transduction pathways, beginning at the cell surface and continuing to the nucleus. It functions in antigen receptor signaling, cytokine responses, Fas-mediated cell death, regulation of gene transcription, and in so many other cases that it clearly constitutes a major mechanism in cell response regulation. Here we demonstrate that it also operates in the response to MCP-1 and presumably to chemokines in general. As indicated earlier (29), the implication of the CCR2 N-terminal domain in MCP-1 binding suggests a "pseudo-tethered" ligand for MCP-1 activation, such that the N-terminal domain would bind MCP-1 with high affinity, whereas another domain would be involved in receptor triggering. In addition, MCP-1 is presumed to be monomeric and has been reported to bind to its receptor in a 1:1 complex (30). As has been demonstrated for other receptors (31), therefore, CCR2 receptor dimerization may require the formation of a stable 1:1 MCP-1/CCR2 intermediate complex, in which an MCP-1 molecule would bind to the CCR2 receptor through a high-affinity site (N-terminal) and a low-affinity site (third extracellular domain), promoting conformational changes that favor JAK2 activation and dimer formation.

The existence of a putative dimerization motif (**G**XXX-**L**XX**L**) in the CCR2 N-terminal and transmembrane I regions, similar to that found in the transmembrane VI of the $\beta$-adrenergic receptor (20), strongly suggests a role for this region in CCR2 dimerization. Although we cannot exclude the possibility that dimerization occurs with the involvement of adapter molecules, it appears unlikely, as GPCR dimer formation using purified receptors recently has been demonstrated for the $\beta$-adrenergic (20) and the $\delta$-opioid receptors (32). Furthermore, Benkirane *et al.* (33) have shown that CCR5 may exist as a dimer even in the absence of ligand stimulation, and that dimer formation is related to susceptibility to HIV type 1 infection. The functional significance of this dimerization also was suggested by Hebert *et al.* (20) using the epitope tagging approach, with which they demonstrated that agonist stimulation of the $\beta$2-adrenergic receptor stabilizes the dimeric state of the receptor. These data clearly show that GPCR dimerization is implicated in the signaling transduction pathway mediated through this receptor. Chemokine-induced GPCR dimerization does not occur only in the CCR2 receptor, as RANTES and SDF-1$\alpha$ also trigger CCR5 and CXCR4 dimerization, respectively (unpublished work). This dimerization model provides a context for understanding the ability of chemokines to trigger chemotaxis. Indeed, the ability of bacteria to sense chemical attractants by a very similar mechanism recently has been described (34); there, it was postulated that the ligand induces changes in the signaling activity by triggering a cluster of receptors by oligomerization. We conclude that these results identify a molecular mechanism that may underlie chemokine responses, revealing additional possibilities for the development of novel therapeutic alternatives for inflammation as well as for AIDS.

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