Dissociation of Chemotaxis from Agonist-induced Receptor Internalization in a Lymphocyte Cell Line Transfected with CCR2B

EVIDENCE THAT DIRECTED MIGRATION DOES NOT REQUIRE RAPID MODULATION OF SIGNALING AT THE RECEPTOR LEVEL

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To investigate the role of the carboxyl-terminal region (52 amino acids) of the monocyte chemoattractant protein 1 receptor (CCR2B) in chemotaxis, we created a series of mutants and expressed them in a murine pre-B lymphocyte cell line. Truncation of the cytoplasmic carboxyl tail to 20 amino acids had little or no effect on chemotaxis or signal transduction, but further truncation resulted in marked functional defects. Upon incubation with monocyte chemoattractant protein 1, CCR2B underwent rapid and extensive internalization, and this was impaired progressively as the carboxyl tail was truncated from 52 to 8 amino acids. Mutation of all of the serine and threonine residues in the carboxyl tail to alanine also resulted in markedly impaired receptor internalization but did not affect signaling or chemotaxis. We conclude that the membrane-proximal portion of the cytoplasmic carboxyl tail of CCR2B is critically involved in chemotaxis and signal transduction, but neither phosphorylation of carboxyl serines or threonines nor internalization of the receptor is required for robust chemotaxis.

Chemokines (chemotactic cytokines) are small, basic peptides that induce directed migration in leukocytes (for reviews, see Refs. 1–4). The chemokines can be subdivided into two families, based on the positions of the first two cysteines and on the quaternary structure of chemokine homodimers (5, 6). Monocyte chemoattractant 1 (MCP-1) is a member of the C or β chemokine family, in which the first two cysteines are adjacent, and is a potent chemoattractant for monocytes, basophils, and memory T cells. Chemokine receptors are members of the seven-transmembrane domain family of receptors and mediate leukocyte activation by coupling to G-proteins. We have shown previously that CCR2A and CCR2B, the chemokine receptors for MCP-1, signal through multiple G-proteins, including Goi, Goq, and Gα16 (7). In contrast to other seven-transmembrane domain receptors in which an extended third intracellular loop (50–75 amino acids long) interacts directly with G-proteins, the chemokine receptors are characterized by extremely short third loops (12 amino acids in the case of CCR2), and thus other portions of these receptors, such as the carboxyl-terminal domain, may be critically involved in signal transduction (8). In the case of CCR2, phosphorylation of serine and threonine residues in the carboxyl tail has also been shown to mediate rapid receptor deactivation (8).

Chemotaxis is the prototypic function of the chemokines, but the signal transduction pathways utilized to initiate directed cell migration are not well understood. Moreover, accurate tracking of changing chemokine gradients may require rapid modulation of signaling at the level of the receptor. In the present study, we have created a series of CCR2B mutants to test the hypothesis that receptor deactivation is required for chemotaxis.

EXPERIMENTAL PROCEDURES

Reagents—MCP-1 was obtained from R&D Systems (Minneapolis). LipofectAMINE, RPMI 1640, and minimal essential medium with Earle’s balanced salt were from Life Technology, Inc. M1 antibody was obtained from Eastman Kodak Co. Fetal calf serum was from Hyclone Laboratories (Logan, UT). [γ-32P]ATP and [3H]adenine were purchased from NEN Life Science Products.

DNA Constructs—Truncation mutants of CCR2B were generated by polymerase chain reaction using the wild-type receptor as a template (9). Downstream primers were designed to introduce a stop codon as well as an NcoI restriction site. An upstream primer was located at ~10 base pairs 5’ to an internal EcoRI site in CCR2B. Mutation of serine/threonine residues to alanine was also done by polymerase chain reaction as described (8). All constructs incorporated the Flag epitope and prolactin signal sequence at their 5’ end as described previously (8), were subcloned into the vector pCMV-1 (10), and were sequenced completely before use.

Cell Culture and Transfection—Human embryonic kidney (HEK)-293 cells were obtained from the American Type Tissue Culture Collection (CRL 1573, Bethesda, MD) and were grown in minimal essential medium with Earle’s balanced salt solution supplemented with 10% fetal calf serum, streptomycin (100 μg/ml), and penicillin (100 IU/ml) at 37 °C in 5% CO2. cDNAs were transfected using LipofectAMINE according to the manufacturer’s instructions, and stable cell lines were obtained by selection in G418. 300-19 pre-B cells (11) were a generous gift from Dr. G. La Rosa (LeukoSite, Cambridge, MA) and were cultured in RPMI 1640 supplemented with 10% fetal calf serum, streptomycin (100 μg/ml), and penicillin (100 IU/ml), glutamine (2 mM), and β-mercaptoethanol (50 μM). Transfection of 300-19 cells was done by electroporation (Bio-Rad). Briefly, 2 × 106 cells in 400 μl of medium were mixed with 30 μg of the cDNAs in expression vectors and 1.5 μg of a plasmid that conferred resistance to neomycin (pSV2-neo) and were maintained at 4 °C for 15 min. Electroporation was performed at 350 V and 960 microfarads. After an overnight incubation, positive clones were selected in the presence of 800 μg/ml G418.

Fluorescence-activated Cell Sorter (FACS) Analysis—A pool of G418-resistant cells was analyzed for cell surface expression of receptors by a FACS. Approximately 3 × 106 harvested cells were incubated at room
temperature for 1 h with culture medium containing phycocerythrin-conjugated M1 antibody. Unbound antibody was removed by washing with phosphate-buffered saline (PBS), and the cells were reusessed in PBS plus 20 μM propidium iodide.

Cytokines and Binding Assays—MCP-1 (5 μg) was labeled using the Bolton-Hunter reagent (diiodide, NEN Life Science Products) as described previously (12). Unconjugated iodide was separated from labeled protein by elution through a PD-10 column (Pharmacia Biotech) equilibrated with PBS and bovine serum albumin (BSA) (1% w/v). Specific activity was determined by immunosassay (Quintikine; R&D) of the labeled protein and by counting gamma emissions and was typically 2,200 Ci/mmol. Equilibrium binding was performed as described (12). Briefly, 35S-labeled ligand, with or without a 100-fold excess of unlabeled ligand, was added to 0.5 × 10⁶ cells in polypropylene tubes in a total volume of 300 μl (50 mM HEPES, pH 7.4, 1.0 mM CaCl₂, 5.0 mM MgCl₂, 0.5% BSA) and incubated for 90 min at 37 °C on an orbital shaker set at 150 rpm. The cells were collected on glass-fiber filters presoaked in 0.3% polyethylimine and 0.2% BSA with a Skatron cell harvester (Skatron Instruments, Sterling, VA). Unbound ligand was removed by washing with 4 ml of buffer (10 mM HEPES, pH 7.4, 500 mM NaCl, 0.5% BSA) for 10 s. Competition with unlabeled ligand was determined by incubation of 0.5 × 10⁶ transfected cells (as above) with 1.5 μM radiolabeled ligand in a final volume of 300 μl. The samples were collected, washed, and counted as above. The data were analyzed with the curve-fitting program Prism (GraphPad, San Diego) and the iterative nonlinear regression program LIGAND.

Ca²⁺ Measurement—Agonist-dependent increases in cytoplasmic Ca²⁺ were determined in transfected 300-19 cells as described previously (13).

Adenyl Cyclase Assay—HEK-293 cells stably transfected with wild-type and truncated CCR2B were grown until subconfluent in 12-well dishes and labeled overnight with 2 μCi/ml [3H]adenine (25–30 Ci/mmol) in minimal essential medium with 10% fetal calf serum. The next day, the cells were washed with serum-free medium supplemented with 1 mg/ml BSA and 10 mM HEPES. After removal of the washing medium, the cells were stimulated by the addition of fresh medium containing 50 μM forskolin and the indicated concentrations of MCP-1 for 5 min. After incubation, Laemml sample buffer was added (15), followed by electrophoresis and visualization of the phosphoproteins by autoradiography. A PhosphoImager (Fuji Medical Systems, Stamford, CT) was used to quantify band intensity.

Assessment of Surface Expression and Internalization of the Receptor—The surface expression of CCR2B was assessed by enzyme-linked immunosorbent assay as described previously (16). Briefly, 6 × 10⁶ cells/tube were washed twice with PBS and then incubated with 1 μg/ml anti-M1 antibody in RPMI 1640 containing 0.1% BSA for 1 h at 37 °C. The cells were then incubated with or without 50 nM MCP-1 for various times at 37 °C and fixed with 3% paraformaldehyde at 4 °C for 15 min. After washing with PBS, the cells were incubated with horseradish peroxidase-conjugated second antibodies (Bio-Rad; 1:10,000 dilution in RPMI 1640, 0.1% BSA) for 30 min at room temperature and washed once again with PBS. Color development was initiated by the addition of 1 mg/ml 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma) in citrate/phosphate buffer, pH 4.0, with 0.03% hydrogen peroxide. Absorbance at 450 nm was measured with an enzyme-linked immunosorbent assay plate reader (Vmax, Molecular Devices, Menlo Park, CA). The rate of internalization was expressed as a ratio of receptor expression in the presence or absence of MCP-1.

Chemotaxis Assays—The migration of 300-19 cell lines was determined using a modification of the method of Campbell et al. (17). Briefly, 300-19 cells stably transfected with mutated or wild-type CCR2B were resuspended in RPMI 1640 plus BSA (1 mg/ml). Cell density was adjusted to 5 × 10⁵ cells/ml, and 5 × 10⁵ cells were added to the top chamber of a 24-transwell apparatus (6.5-mm diameter, 5-μm pore size, Costar 3421, Corning Costar, Cambridge, MA) and incubated for 3 h at 37 °C in an atmosphere containing 5% CO₂. Cells that passed through the membrane were collected from the lower well and counted in a FACScan (Becton-Dickinson, San Jose, CA). Chemotaxis was distinguished from chemokinesis by “checkerboard” analysis as described by Zigmond and Hirsch (18). Cell numbers, determined with a Coulter counter (Coulter, Miami, FL), were in excellent agreement with the results of the FACScan analysis.

RESULTS

Expression of CCR2B Truncation Mutants in Transfected Cells—To examine the role of the carboxyl-terminal domain of CCR2B in chemotaxis and signaling, we created a series of four mutants, which this region of 52 amino acids was truncated progressively to 33, 27, 20, and finally 8 amino acids. These constructs were designated CCR2A1, CCR2Δ2, CCR2Δ3, and CCR2Δ4, respectively (Fig. 1). Because phosphorylation of carboxyl tail serine and threonine residues is known to contribute to the desensitization of CCR2 (8), we created two additional mutants, CCR2-ALA contains the full-length carboxyl tail in which all of the serine and threonine residues (a total of 10)
were changed to alanines. In CCR2Δ3-ALA, the serine and threonine in CCR2Δ3 were changed to alanine. Stably transfected cell lines were established in a murine pre-B lymphocyte cell line (300-19) (11), and analysis by flow cytometry revealed cell surface expression of the six variant forms comparable to that of wild-type CCR2 (Fig. 2).

**Binding of MCP-1 to CCR2B Truncation Mutants**—We determined previously that the amino-terminal region of CCR2B is the high affinity binding site for MCP-1 (12). In equilibrium binding studies, each of the carboxyl tail truncation mutants bound 125I-labeled MCP-1 with an affinity similar to that of wild-type CCR2B (Table I). An intact cytoplasmic carboxyl tail, therefore, is not required for high affinity binding of MCP-1 to CCR2B.

**Intracellular Calcium Mobilization**—Because the CCR2B truncation mutants bound MCP-1 with high affinity, we next assessed their ability to initiate signal transduction. We first examined agonist-dependent mobilization of intracellular Ca2+ in the 300-19 cell transfectants. In response to a saturating concentration of MCP-1, a robust calcium flux was observed in cells expressing CCR2Δ1, CCR2Δ3, and the two alanine mutants (Fig. 3A). CCR2Δ2 was not studied in detail because the more severely truncated CCR2Δ3 was essentially identical to CCR2Δ1. CCR2Δ4, which retained only eight carboxyl tail residues, had a significantly diminished maximal response. Dose-response studies of these constructs over a broad range of MCP-1 concentrations confirmed that all, except CCR2Δ4, had EC50 values very similar to that of the wild-type receptor (Fig. 3B and Table I). Consistent with published findings (8), mutation of CCR2 carboxyl tail serines and threonines, or loss of these residues as a result of receptor truncation, led to a prolongation of the rise in intracellular calcium (Fig. 3B). Chelation of extracellular calcium with EGTA did not affect either the magnitude or the prolongation of the calcium response in the truncation mutants (data not shown).

**Inhibition of Adenylyl Cyclase**—CCR2B couples to Gαi to lower cAMP levels (7, 13), and as a second assay of signal transduction, we determined the ability of each mutant to inhibit adenylyl cyclase. Except for CCR2Δ4, the truncation mutants were as active as wild-type CCR2B in this assay (Fig. 4). As summarized in Table I, the IC50 for CCR2Δ4 was approximately 25-fold higher. These experiments, which were performed in a different cell type (HEK-293 cells), recapitulated the results of the calcium flux studies in 300-19 cells.

**Activation of Mitogen-activated Protein Kinase**—In contrast to the results of the calcium flux and adenylyl cyclase experiments, CCR2Δ4 was as potent as wild-type CCR2B or the longer truncation mutants in activating ERK (Fig. 5). These data indicate that the carboxyl tail of CCR2B does not play a critical role in activation of the mitogen-activated protein kinase pathway.

**Agonist-dependent Receptor Internalization**—In response to MCP-1, CCR2B was internalized rapidly, with 60–70% of the wild-type receptor removed from the cell surface by 15 min (Fig. 6). Truncation of the carboxyl tail resulted in a progressive slowing of internalization, such that more than 75% of CCR2Δ4, CCR2Δ3, Δ3-ALA, and CCR2-ALA remained on the cell surface at 15 min. The internalization of the truncation mutants could be roughly divided into three groups: Δ4 was the most impaired; Δ3, Δ3-ALA, and CCR2-ALA had an intermediate level of impairment; and Δ2 and Δ1 were internalized almost as well as the wild-type receptor. These data suggest that multiple regions of the carboxyl tail and, in particular, the amino acids between 316 (CCR2Δ4) and 328 (CCR2Δ3) play important roles in agonist-dependent receptor internalization. The data further indicate that neither the serine nor the threonine in CCR2Δ3 plays a critical role in receptor internalization, since mutation of both to alanine (Δ3-ALA) did not change the rate of internalization compared with Δ3.

**Chemotaxis**—Finally, we examined each of the mutants for its ability to induce chemotaxis when stably expressed in 300-19 cells. As shown in Fig. 7, cells transfected with CCR2B exhibited a classical biphasic response to MCP-1. The MCP-1 concentration for optimal chemotaxis was 1 nM, and the chemotactic index (ratio of cells migrating in the presence versus the absence of MCP-1) was in excess of 5,000. Checkerboard analysis confirmed that virtually all of this response was caused by chemotaxis and not chemokinesis (data not shown). Except for Δ4, a similar pattern was seen with each of the truncation mutants, although the chemotactic indices tended to be lower. In Δ1, the peak response was similar to that in the wild-type receptor but was left-shifted to 0.1 nM. In cells ex-
pressing Δ4, the chemotactic index was clearly lower, the curve was also right-shifted, and the optimal response occurred at approximately 10 nM MCP-1. Once again, checkerboard analysis confirmed that this response was not caused by chemokine-sis (data not shown).

**DISCUSSION**

We have shown previously that the cytoplasmic carboxyl terminus of CCR2B plays an important role in receptor deactivation and internalization (8). To determine if this region of the receptor is critically involved in chemotaxis, we created a series of progressive truncations of the carboxyl domain and also mutated all serine and threonine residues to alanine. There are two important results of this study. First, the 12 amino acids between CCR2D3 (Phe-328) and CCR2D4 (Leu-316) are critically involved in signal transduction, agonist-dependent internalization, and chemotaxis. Second, rapid deactivation of the receptor, either by agonist-induced phosphorylation of carboxyl domain serine and threonine residues or by receptor internalization, is not required for chemotaxis. To our knowledge, this is the first demonstration of a dissociation of receptor internalization/desensitization and chemotaxis.

Chemotaxis is the prototypic function of the chemokine receptors, yet little is known of the signaling pathways and receptor domains involved in this complex phenomenon. Unlike

**FIG. 3.** Agonist-dependent calcium mobilization in 300-19 cells expressing wild-type and truncated CCR2B. Stably transfected 300-19 cells were loaded with Indo-1 AM, and intracellular calcium levels were measured as described under “Experimental Procedures.” Panel A, calcium flux in response to MCP-1 (100 nM). Shown is a representative experiment (n = 3). Panel B, dose response to MCP-1. The EC50 values are given in Table I. Data are the means of at least three independent experiments. See key in legend to Fig. 2.

**FIG. 4.** Inhibition of adenylyl cyclase in wild-type and truncated CCR2B. HEK-293 cells expressing CCR2B or the indicated truncation mutants were labeled with [3H]adenine and stimulated with 10 μM forskolin in the presence or absence of MCP-1. [3H]cAMP pools were measured as described under “Experimental Procedures.” Data shown are the means of three independent experiments.

**FIG. 5.** ERK2 activation induced by MCP-1. 300-19 cells stably expressing CCR2B or CCR2B truncation mutants were maintained in the absence of serum for 24 h, incubated with or without 10 nM MCP-1 for 5 min, and lysates were collected to assay for ERK activity as described under “Experimental Procedures.” Phosphorylation of myelin basic protein was in the absence or presence of MCP-1. C denotes the absence of MCP-1 in control cells; M denotes the presence of MCP-1 (10 nM). Data are representative of three independent experiments. The ERK content of the lysates, as determined by Western blotting, was similar for all receptor variants (lower portion of the panel). Wt, CCR2B wild-type; UT, untransfected.

**FIG. 6.** Agonist-dependent internalization of CCR2B and its mutants in 300-19 cells. Stably transfected 300-19 cells were labeled with the M1 antibody for 1 h and then incubated in the presence or absence of MCP-1 (50 nM) for the indicated times at 37 °C. Surface expression of the receptors was measured as described under “Experimental Procedures.” Data are the ratio of receptor expression in the presence and absence of MCP-1 and are the means of at least four experiments.
freshly isolated blood cells, transfected cells do not generally show robust chemotactic responses (19), and this has been a significant problem in the chemokine field. Our studies have utilized a modification of the chemotaxis assay described by Campbell et al. (17). Stable lines of wild-type and mutated CCR2B were established in a pre-B lymphocyte cell line (300-19) and yielded a chemotactic index (for the wild-type receptor) in excess of 5,000, as opposed to 15 for transfected HEK-293 cells (19). Using this assay, we have found that truncation of CCR2B to within 8 amino acids of the membrane (CCR2Δ4) does not abolish chemotaxis but does result in a reduced chemotactic index as well as a shift to higher MCP-1 concentrations for optimal cell migration. The reduction in chemotaxis correlated well with reduced signal transduction in the calcium flux and cyclase assays and is thus most simply explained as a direct consequence of poor coupling of the truncated receptor to G-proteins. That the most severely truncated mutant (CCR2Δ4) signaled well in the mitogen-activated protein kinase assay most likely reflects the multiple rounds of signal amplification between ligand binding and ERK activation.

CCR2Δ4 internalized much more slowly than the wild-type receptor, and its ability to mediate chemotaxis was also markedly impaired. This result raised the question of whether receptor sequestration is necessary for robust chemotaxis. To address this issue, we created a second CCR2 mutant, CCR2-ALA, in which all 10 of the carboxyl tail serine and threonine residues were mutated to alanine. Agonist-dependent internalization of seven-transmembrane domain receptors is in large part dependent upon phosphorylation of these serine and threonine residues (for review, see Ref. 20), and as expected, CCR2-ALA internalized very slowly. The chemotactic response, however, was fully as robust as that of the wild-type receptor. In addition, a number of other slowly internalizing mutants (CCR2Δ3, CCR2Δ3-ALA) were similar to the wild-type CCR2B in their chemotactic response. We conclude that receptor internalization is not an important component of the chemotactic response, at least as measured in this unidirectional assay. Further, because CCR2-ALA cannot undergo rapid desensitization (8), we further conclude that neither of the these two well described mechanisms of receptor deactivation is critically involved in mediating chemotaxis. Rather, these results suggest that once activated, the cell migrates in a process that does not require continuous modulation at the level of the receptor. Whether receptor internalization and desensitization are important for a migrating cell to change direction (i.e. follow a changing gradient) remains to be determined.

Truncation of the carboxyl-terminal domain of CCR2B to 8 amino acids markedly impaired signal transduction. The cytoplasmic carboxyl tails of seven-transmembrane domain receptors have been implicated in G-protein coupling. Liggett et al. (21) reported that the carboxyl portion of the third intracellular loop and, to a lesser extent, the membrane-proximal region of the carboxyl tail participated in the coupling of the β2-adrenergic receptor to Gas. Similarly, Cotecchia et al. (22) found that substitution of the most membrane-proximal amino acids of the carboxyl tail of the α1-adrenergic receptor with the corresponding region of the β2-adrenergic receptor resulted in a marked decrease in agonist-dependent activation of phospholipase C. More recently, Namba et al. (23) showed that differentially spliced isoforms of the carboxyl-terminal domain of the prostaglandin E receptor couple to different G-proteins. In the current study, G-protein coupling was assessed by determining agonist-dependent inhibition of adenyl cyclase in transfected epithelial HEK-293 cells, as well as by intracellular calcium elevation in the hematopoietic 300-19 cells. In both cell types, our data indicate an important role for the membrane-proximal amino acids of the CCR2 carboxyl tail (i.e. amino acids 316–328) in coupling to G-proteins.

Truncation of the cytoplasmic carboxyl tail also led to a progressive impairment in the ability of the mutants to undergo agonist-dependent internalization. That CCR2Δ4 was clearly more impaired in its ability to internalize than CCR2-ALA also indicates that carboxyl tail residues other than the 10 serines and threonines play a role in receptor sequestration. Further evidence for this point is derived from the fact that CCR2Δ3 and CCR2Δ3-ALA had virtually identical phenotypes in terms of receptor sequestration.

Cysteine residues in the carboxyl tails of the G-protein-coupled receptors are potential sites for palmitoylation and may serve to form a fourth intracellular loop (22, 24). It is not known if either of the two cysteines in this region of CCR2B (329 and 332) is palmitoylated. However, CCR2Δ3 (F328) lacks both of these cysteines, and yet was very similar to the wild-type receptor in signaling and chemotaxis assays. The cysteine residues in the carboxyl tail of CCR2B, therefore, do not appear to be critical for receptor function.

To our knowledge, only one other study has examined the role of the carboxyl-terminal domain of a chemokine receptor in chemotaxis (19). In that study, truncation of the carboxyl tail of CXCR2 to 6 amino acids completely abolished chemotactic activity, and the authors postulated a role for this receptor domain in mediating chemotaxis. However, because other measurements of signal transduction were not performed, it was equally likely that the truncated receptor was simply uncoupled from G-proteins. Data in the current study support this latter view and further suggest that normal mechanisms of rapid receptor deactivation, such as agonist-induced sequestration and desensitization, are not involved in chemotaxis.

In summary, we have shown that the 12 amino acids between leucine 316 and phenylalanine 328 of the CCR2B carboxyl domain play an important role in chemotaxis, signal transduction, and agonist-dependent receptor sequestration. Neither agonist-induced receptor internalization nor phosphorylation of carboxyl tail serine/threonine residues appears to be critically involved in any of these responses, including chemotaxis. Further elucidation of this pathway may identify targets for pharmacological intervention in the treatment of inflammation.

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FIG. 7. Chemotaxis of 300-19 cells expressing CCR2B and CCR2B mutants. MCP-1-dependent chemotaxis was measured as described under “Experimental Procedures.” Data are the means ± S.E. from at least six independent experiments. Panel A, CCR2B-Wt, Δ1, Δ2, Δ3, and Δ3ALA. Panel B, CCR2B-Wt, ALA, Δ4, and untransfected 300-19 cells. Wt, CCR2B wild-type.
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