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References

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The Chemokine Monocyte Chemotactic Protein 1 Triggers Janus Kinase 2 Activation and Tyrosine Phosphorylation of the CCR2B Receptor

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The chemokines are a growing family of low m.w., 70- to 80-residue proinflammatory cytokines that operate by interacting with G protein-coupled receptors. Chemokines are involved in cell migration and in the activation of specific leukocyte subsets. Using the Mono Mac 1 monocyctic cell line, we show that monocyte chemotactic protein 1 (MCP-1) triggers activation of the Janus kinase 2 (JAK2) and STAT3 pathway and CCR2 receptor tyrosine phosphorylation. Both Ca2+ mobilization and cell migration are blocked in Mono Mac 1 cells by tyrphostin B42, a specific JAK2 kinase inhibitor. Within seconds of MCP-1 activation, JAK2 phosphorylates CCR2 at the Tyr139 position and promotes JAK2/STAT3 complex association to the receptor. This MCP-1-initiated phosphorylation and association to JAK2 is also observed in CCR2B-transfected HEK293 cells. In contrast, when a CCR2B Tyr139Phe mutant is expressed in HEK293 cells, it is not phosphorylated in tyrosine and triggers neither JAK2/STAT3 activation nor Ca2+ mobilization in response to MCP-1. These results implicate the tyrosine kinase pathway in early chemokine signaling, suggesting a key role for this kinase in later events. *The Journal of Immunology, 1998, 161: 805–813.

Lymphocyte migration to inflammation sites is a multistep process mediated by a series of sequential, but overlapping, interactions in which the generation of chemotactic gradients plays a key role. Chemotactic cytokines, or chemokines, are responsible for chemotraction of specific subsets of leukocyte populations to inflammation sites (1, 2).

The chemokines are a growing family of low m.w., 70- to 80-residue proinflammatory cytokines, characterized by their ability to induce migration and activation of specific leukocyte population subsets (2–6). The two main branches of the family are distinguished by overall sequence homology, chromosomal location, and leukocyte population specificity (7). The CXC (α) chemokines act on neutrophils and nonhemopoietic cells involved in wound healing, whereas the CC (β) chemokines act on monocytes, eosinophils, basophils, NK cells, and different lymphocyte subpopulations (5, 6). New chemokine families have been identified recently, which maintain overall sequence homology but lack the typical cysteine distribution (C, or γ, and Cx,C, or δ, chemokines) (8, 9).

The CC chemokine monocyte chemoattractant protein 1 (MCP-1) was originally described as a potent chemoattractant for monocytes (10), produced by different cell types in response to a variety of mediators including PDGF (platelet-derived growth factor), TNF-α, LPS, and oxidized low density lipoproteins (11, 12). Since then, many other activities have been assigned to MCP-1, including induction of T cell migration (13), suppression of tumor growth in animal models (14), and neutralization of HIV-1 (15). MCP-1 has also been associated with several disease states in which monocyte infiltration plays a role, such as granulomatous disease, host responses to bacteria, rheumatoid arthritis, inflammatory heart disease, bone trauma, asthma, and sepsis (16, 17).

Chemokines activate their functions through interaction with single-chain, seven-helix membrane-spanning receptors coupled to G proteins (GPCR) (3). To date, nine receptors have been defined for the CC chemokines (18) and four for the CXC family, together with several putative CC or CXC receptors for which the ligands remain to be determined. They have an N terminus and three extracellular loops that act in concert to bind the chemokine ligand (19, 20), while the three intracellular loops and the C terminus are involved in transduction of the chemokine signal. The activation signals following chemokine stimulation are not well defined, and various signal transduction pathways have been implicated through the association of the chemokine receptors with guanine nucleotide-binding protein (G proteins) (21). In all cases, a pertussis toxin (PTX)-sensitive G protein was found, although there are also examples of PTX-insensitive G proteins, as is the case for the C5a receptor, which also couples to Go16 (22). The biochemical signaling pathways include changes in intracellular cAMP levels (23), phospholipase activation (24), increases in tyrosine phosphorylation including the Src substrates focal adhesion kinase (p125FAK) and ZAP-70 (25), increased association of Src family

4 Abbreviations used in this paper: MCP-1, monocyte chemoattractant protein 1; CTX, cholera toxin; EGF, epidermal growth factor; GPCR, G protein-coupled receptor; JAK, Janus kinase; PTX, pertussis toxin; PTyr, phosphorytrosine.
kinases with p21ws (26), and activation of the MAPK (mitogen-activating protein kinase) cascade (27).

The cellular response resulting from the activation of a given chemokine receptor can vary depending on the cell in which it is expressed. This is presumably due to the availability of specific G protein subunits and other downstream effector molecules. It has recently been demonstrated that the association of chemokine receptors to different G proteins depends on the receptor and the cell line studied (28).

Biochemical analysis of chemokine signaling has been hampered by the low levels of receptor expression and by the promiscuity of individual receptors with various ligands. Using the CCR2 chemokine receptor as a model, we have analyzed early signaling events activated by MCP-1 binding in a human monocyte cell line (Mono Mac 1). MCP-1 triggers tyrosine phosphorylation and activation of the JAK2/STAT3 pathway in a PTX-independent manner. This MCP-1-initiated phosphorylation and association to JAK2 is also observed in CCR2B-transfected HEK293 cells. In contrast, when a CCR2B Tyr139 Phe mutant is expressed in HEK293 cells, it is not phosphorylated in tyrosine and triggers neither JAK2/STAT3 activation nor Ca2+ mobilization in response to MCP-1. Altogether, these data indicate that within seconds of activation, JAK2 phosphorylates CCR2 at the Tyr139 position and promotes JAK2/STAT3 complex association to the receptor. The blockage of MCP-1-induced Ca2+ mobilization and cell migration in Mono Mac 1 cells treated with the specific JAK2 kinase inhibitor, tyrphostin B42 (29), demonstrates that JAK2 activation is one of the first events following ligand binding and assigns JAK2 a critical upstream role for all other signaling events, including the association between the receptor and the G proteins.

Materials and Methods

Biologic materials

Mono Mac 1 (DSM ACC252) and HEK293 cells (ATCC TIB202) were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and the American Type Culture Collection (ATCC, Manassas, VA), respectively. Abs used include monoclonal anti-PTyr (4G10) and rabbit anti-JAK2 (Upstate Biotechnology, Lake Placid, NY); anti-Gαi, anti-STAT3, and anti-STAT5 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-PTyr (PY20) (Transduction Laboratories, Lexington, KY); anti-CD4 (HP2.6; kindly donated by Dr. A. C. Carrera), and anti-GRB2 class 1 (W6/32, ATCC). Anti-CCR2 mAb MCP-1R05 and MCP-1R05 were generated in our laboratory (15, 30). Epidermal growth factor (EGF)-stimulated A-431 cell lysates were from Upstate Biotechnology and MCP-1 from Peprotech (London, U.K.).

Flow cytometry analysis

Cells were centrifuged (250 × g, 10 min, room temperature), plated in V-bottom 96-well plates (2.5 × 106 cells/well) and incubated with 50 μl well biotin-labeled mAb (5 μg/ml, 60 min, 4°C). Cells were washed twice in PBS with 2% BSA and 2% FCS and centrifuged (250 × g, 5 min, 4°C), FITC-labeled streptavidin (Southern Biotechnologies, Birmingham, AL) was added, cells incubated (30 min, 4°C), and plates washed twice. Cell-bound fluorescence was determined in a Profile XL flow cytometer at 525 nm (Coulter Electronics, Miami, FL).

Calcium determination

Changes in intracellular calcium concentration were monitored using the fluorescent probe Fluo-3, AM (Calbiochem, La Jolla, CA). Cells (2.5 × 106 cells/ml), untreated or treated for 16 h with either cholera toxin (CTX; 0.4 μg/ml) or PTX (0.1 μg/ml), were resuspended in RPMI containing 10% FCS and 10 mM HEPES and incubated with 10 μM Fluo-3 AM (300 μM in DMSO) for 15 min at 37°C. After incubation, cells were washed and resuspended in complete medium containing 2 mM CaCl2 and maintained at 4°C until just before MCP-1 addition, to minimize membrane trafficking and to eliminate spontaneous Ca2+ entry. Calcium mobilization in response to 5 nM MCP-1 (Peprotech) was determined at 37°C in an EPICS XL flow cytometer at 525 nm (Coulter) and included background level stabilization and determination of the level of probe.

FIGURE 1. MCP-1 induction of Ca2+ mobilization and Gαi association to CCR2 receptor in Mono Mac 1 cells. A, 5 nM MCP-1-induced Ca2+ mobilization in Mono Mac 1 cells, untreated or preincubated with CTX or PTX, was determined at 525 nm in a flow cytometer. The result of one of three experiments performed is shown. Results are expressed as percentage of the maximum chemokine response. Equivalent Fluo-3, AM loading was determined as indicated in Materials and Methods. B, 5 nM MCP-1-induced Mono Mac 1 cell lysates were immunoprecipitated with anti-CCR2 (MCP-1R03), or anti-CD4 (HP2.6) Abs as control, and the Western blot developed with anti-Gαi, Ab. As a control for the presence of equal amounts of CCR2 in the different lanes, the blot was stripped and developed with the anti-CCR2 Ab MCP-1R05. The arrow indicates the position of Gαi.
Mono Mac 1 cells (0.5 \times 10^6 cells/ml), untreated or preincubated with 25 μM tyrphostin B42 or A1 (Calbiochem-Novabiochem, La Jolla, CA) for 12 h at 37°C as described (29).

Transfection

Human HEK293 embryonic kidney cells were transfected with human CCR2B and CCR2BY139F cDNA cloned in pcDNAIII by the calcium phosphate precipitation method. Stably transfected cells were selected in G-418 (Life Technologies, Gaithersburg, MD) and screened in flow cytometry analysis for receptor expression using anti-CCR2 Abs. All experiments were performed using at least four cell lines for each plasmid.

Site-directed mutagenesis of CCR2B Tyr139

Phenylalanine was substituted for tyrosine at residue 139 in the CCR2B receptor by overlap extension. Briefly, two primary PCR with overlapping bases. For secondary PCR, both primary fragments were end-repaired within 30 s of MCP-1 triggering, and persists for 5 min after chemokine binding. As occurs in other cell systems, the CCR2 receptor, therefore, regulates calcium release and couples to the G protein in Mono Mac 1 cells.

MCP-1 induces tyrosine phosphorylation of the CCR2 receptor

In MCP-1-activated Mono Mac 1 cells, a 38-kDa protein phosphorylated in tyrosine residues was initially identified as the CCR2 receptor (32). It then became clear that some MCP-1 downstream signals in other monocyte lines and T cells are coupled to PTX-sensitive G proteins (28). Using a CCR2-specific mAb, MCP-1R03 (15, 30), we analyzed the physical association between the CCR2 receptor and the G protein, therefore, regulates calcium release and couples to the G protein in Mono Mac 1 cells.
developed with anti-PTyr Ab (Fig. 2, lower panel), while no differences were observed in the amount of CCR2 in each lane (Fig. 2, lower panel) when the same membrane was stripped and reblotted with anti-CCR2 Ab. The same phosphorylated 38-kDa band was observed in both cases, confirming phosphorylation of the CCR2 upon MCP-1 stimulation. An increase in CCR2 receptor phosphorylation is seen as early as 30 s after MCP-1 stimulation, and phosphorylation persists for 20 to 30 min, decreasing thereafter. We have observed residual phosphorylation of CCR2 in untreated cells (Fig. 2), the significance of which is discussed below.

**FIGURE 3.** MCP-1-induced receptor association of JAK2 kinase. A, Mono Mac 1 cells were MCP-1 stimulated (5 nM), and lysates were immunoprecipitated with anti-CCR2 mAb and analyzed in Western blot with anti-JAK2 Ab. Control MCP-1-stimulated (5 nM) Mono Mac 1 cell lysates immunoprecipitated with anti-CD4 and analyzed with anti-JAK2 are shown. As a positive control, EGF-stimulated A431 cell lysates were tested in Western blot with the same anti-JAK2 Ab. CCR2 protein loading was controlled as described in Figure 2 (lower panel). B, PTX-treated Mono Mac 1 cells were MCP-1 stimulated as in A, lysates immunoprecipitated with anti-PTyr (PY20) and analyzed in Western blot with anti-CCR2 mAb. C, PTX-treated Mono Mac 1 cells were stimulated with MCP-1, lysed, and analyzed in Western blot as described for A.

**MCP-1 induces rapid JAK-2 activation and association to the CCR2 in a PTX-independent manner**

To ascertain which kinase is responsible for the rapid CCR2 chemokine receptor phosphorylation, Mono Mac 1 cells were stimulated with MCP-1, and cell lysates were immunoprecipitated with anti-CCR2 or anti-CD4 as an isotype-matched Ab control. Anti-JAK2 Abs identified a 130-kDa protein in the anti-CCR2 immunoprecipitate (Fig. 3A). Furthermore, JAK2 phosphorylated in tyrosine is observed after MCP-1 stimulation (Fig. 4A), while JAK2...
levels are unchanged (Fig. 4A) as determined by stripping and reblotting the same membrane with anti-JAK2 Ab. JAK2 association to the CCR2 receptor takes place as early as 30 s after MCP-1 stimulation (Fig. 3A); small amounts of JAK2 were also found associated to the CCR2 receptor in the absence of added MCP-1, consistent with receptor phosphorylation in the absence of exogenous ligand in Mono Mac 1 cells. Immunoprecipitation of cell lysates with isotype-matched control Abs to CD4 or to other membrane proteins, such as MHC class I, did not reveal the presence of JAK2 (Fig. 3A), ruling out nonspecific protein association to membrane components under our experimental conditions. The rapid association of JAK2 to the CCR2 receptor suggests a role for this tyrosine kinase in early receptor phosphorylation following ligand stimulation. Anti-JAK1 and -JAK3 Abs were used to test for the presence of other JAK family members associated to the CCR2 receptor with negative results (data not shown).

To confirm JAK pathway involvement in the signaling response through the CCR2 chemokine receptor, we analyzed JAK2 kinase-activated STAT transcription factors in anti-CCR2 immunoprecipitates. STAT3, but not STAT5, associates to the receptor complex in response to MCP-1 (Fig. 3A); the association of STAT3 correlates in time with JAK2 phosphorylation and binding. Furthermore, this STAT3 is tyrosine phosphorylated, indicating its activation (Fig. 4C). It thus appears that CCR2 receptor activation induces receptor and JAK2 phosphorylation and STAT3 binding. The MCP-1-triggered tyrosine phosphorylation of the CCR2 receptor is independent of PTX-sensitive G proteins (Fig. 3B); PTX also had no effect on JAK2 kinase binding to the receptor (Fig. 3C). Whereas MCP-1-induced association of JAK2 to the CCR2 receptor is not impeded by this toxin, PTX blocked JAK2 kinase dissociation from the receptor (Fig. 3C), implying that PTX-sensitive G protein activation is important in the recycling of the JAK2/STAT3 receptor complex.

MCP-1-promoted Ca\(^{2+}\) influx and cell mobilization were blocked when cells were pretreated with the specific JAK2 kinase inhibitor, tyrphostin B42 (29) (Fig. 5, A and B), indicating the importance of this kinase in early signaling events. As expected, B42 treatment inhibits G\(\alpha_i\) association to the receptor, in accordance with the role of this G protein in GPCR-mediated effects (Fig. 5C). Tyrphostin treatment was not toxic to Mono Mac 1 cells, as shown by cell cycle analysis using propidium iodide incorporation (not shown).

Despite the rapid and transient JAK2-mediated CCR2 tyrosine phosphorylation, phosphorylated CCR2 can be observed long after maximum JAK2 association, thus implicating other tyrosine kinases, as described for RANTES activation of human T cells (25).
The Tyr\textsuperscript{139} residue of CCR2 is critical in MCP-1-activated signaling

To further investigate the significance of MCP-1-induced CCR2 receptor tyrosine phosphorylation, the CCR2B receptor Tyr\textsuperscript{139}Phe mutant CCR2BY139F was constructed, expressed in HEK293 cells, and its response to MCP-1 analyzed. Tyr139 is located in the second intracellular loop and forms part of the DRY sequence, a highly conserved motif in GPCR that is essential for agonist-mediated receptor activation (35); it is the only intracellular tyrosine conserved in the functionally homologous CCR2A receptor (33). Wild-type and CCR2BY139F mutant receptor expression were identical in stably transfected HEK293 cells as assessed by flow cytometry (Fig. 6, upper panel), and both receptor forms bind MCP-1 equally well (not shown). MCP-1 induces Ca\textsuperscript{2+} mobilization in CCR2B wild-type HEK293 cells, but not in CCR2BY139F mutant or in mock-transfected HEK293 cells (Fig. 6B). Phosphorylation of the mutant receptors was analyzed; in contrast to the wild-type receptor, the CCR2BY139F mutant is unphosphorylated in tyrosine residues following MCP-1 binding (Fig. 7, upper panel) and cannot trigger JAK2 phosphorylation or association to the receptor (Fig. 7, middle panel). Finally, MCP-1 promotes G\textsubscript{a}i association to the CCR2B receptor, but not to the mutant CCR2BY139F receptor (Fig. 7, lower panel). These results clearly demonstrate the relevance of JAK2 phosphorylation, as well as of JAK2 and G\textsubscript{a}i association in the functional response to MCP-1.

Discussion

Transendothelial leukocyte migration into inflammation sites is a complex process in which several cell surface molecules, including the selectin family of adhesion molecules, integrins, and their ligands, act in concert to regulate cell migration. Soluble mediators, mainly chemokines, are also central both in activating and directing specific leukocyte subsets to target tissues. It is known that chemokines bind to GPCR and induce changes in intracellular cAMP levels, activate phospholipases, and increase tyrosine phosphorylation. The initial steps in receptor activation that lead to the wide variety of chemokine-triggered cellular responses are still, however, not known.

MCP-1 was first described as a potent chemoattractant for monocytes and binds the CCR2 and the CCR4 chemokine receptors (36). In CCR2-expressing cells, MCP-1 binding promotes Ca\textsuperscript{2+} mobilization and transmigration, processes that are blocked by PTX treatment but not by CTX (21). Using a human monocytic cell line, Mono Mac 1, which also responds to MCP-1 and CCR2-specific mAb (15, 30), we have demonstrated that G,
associates rapidly (within 30 s) to CCR2 after MCP-1 activation. This confirms previous data indicating that other chemokine receptors, such as IL-8R, bind Gαi in response to IL-8 (37) and extends the observation to members of the CC family of chemokine receptors.

Despite Gαi binding to the CCR2 receptor and its implications in signaling events subsequent to MCP-1 binding, we cannot exclude the participation of other G proteins in the signaling activated by this chemokine. It is reasonable to consider that the cellular response of a given chemokine receptor varies depending on the cell in which it is expressed. This is presumably due to the availability of specific G protein subunits and other downstream effector molecules. It has thus recently been demonstrated that the association of chemokine receptors to different G proteins depends on the receptor and the cell line studied (28).

Chemokines also promote an increase in tyrosine kinase activity; it has recently been shown that RANTES induces activation and assembly of macromolecular focal adhesion complexes and provokes T lymphocyte homotypic adhesion by phosphorylation and association of p125FAK and ZAP-70 (25).

Even more interesting is the identification of the phosphorylated CCR2 receptor itself. Several assays performed to identify the kinase responsible for CCR2 receptor phosphorylation led to the conclusion that JAK2 causes early receptor activation. Both JAK2 association and CCR2 tyrosine phosphorylation occur even in the presence of PTX, indicating no G, participation in this process; JAK2 dissociation was not observed under these conditions, suggesting an active participation of Gi pathways in uncoupling JAK2 from the receptor. In response to MCP-1, this kinase is phosphorylated as soon as 30 s after binding, indicating that JAK2 activation is simultaneous with its association to CCR2.

Furthermore, STAT3, but not STAT5, is associated to the CCR2 following MCP-1 activation in accordance with the role assigned to the JAK tyrosine kinases in transducing signals from hematopoietic growth factor receptors (38). In these receptors, the activation and association of JAK kinase to the receptor creates docking sites for SH2-containing proteins such as STAT, leading to their phosphorylation and activation of gene transcription.

Neither MCP-1-induced, PTX-sensitive G protein-mediated physiologic effects nor Gαi association to CCR2 were observed after treatment of Mono Mac 1 cells with the JAK2-specific inhibitor tyrphostin B42. This is not the case when cells are treated with other tyrphostins, indicating that inhibition of JAK2 kinase activity abolishes the association and activation of the G proteins responsible for this response. This result and the JAK2 association to the CCR2 receptor in PTX-treated cells imply that the first event after MCP-1 binding to the CCR2 in Mono Mac 1 cells is association of the JAK2 kinase. The conformational changes promoted by both ligand interaction and tyrosine kinase association induce Gi protein association to its binding site, probably located in the second intracellular loop, as is the case for the IL-8R (37).

To determine which CCR2B receptor Tyr residue is involved in JAK2 kinase association, and as Tyr139 is the only intracellular tyrosine conserved in the functionally homologous CCR2A receptor (33), we generated a CCR2BY139F receptor by point mutation. Although this mutated receptor binds MCP-1 as well as does the wild-type receptor, it does not elicit MCP-1-triggered functional responses such as Ca2+ mobilization, cell migration, or CCR2B tyrosine phosphorylation. This lack of response is due to abolition of the association to the receptor by the JAK2 kinase and then of the Gi protein. The tyrosine at position 139 is part of the conserved DRY motif present in most chemokine receptors. Earlier studies characterized the critical roles of arginine and aspartic acid in the DRY motif, implicated in G protein-mediated signaling in other

FIGURE 6. CCR2B receptor expression in HEK293 cells. Cells were stably transfected with human CCR2B and mutant human CCR2BY139F cDNA. A, Flow cytometry analysis of anti-CCR2 mAb MCP-1R03 or MCP-1R05 (as indicated) binding to representative CCR2B or CCR2BY139F clones, compared with that of a control mAb (shaded peak). B, MCP-1-induced Ca2+ influx in CCR2B and CCR2BY139F cells was determined by flow cytometry at 525 nm. The result depicts one of three experiments performed with selected clones.
CCR2B tyrosine phosphorylation and JAK2 and Gαi-mediated CCR2 phosphorylation described for HEK293 cell lysate immunoprecipitated with anti-MHC class I Ab and blotted with anti-JAK2 Ab. CCR2 protein loading was controlled by reprobing Western blots with anti-PTyr (not shown).

These data fit a model in which Gαi association to the chemokine is a consequence of conformational changes promoted by both ligand interaction and JAK2 activation. If this is the case, a mutant form with a constitutively associated Gαi protein should activate signaling in response to the chemokine. This occurs, in fact, in another chemotactic peptide receptor belonging to the GPCR family, the C5a receptor (39), in which phenylalanine replaces the tyrosine in the DRY motif, while its capacity to activate Gαi signaling remains intact.

Phosphorylation of the DRY motif tyrosine in a β2-adrenergic receptor has also recently been suggested as linking this receptor to the insulin-like growth factor (IGF)-1 receptor pathway (40). In accordance with recent reports suggesting JAK pathway modulation by other GPCR (41), as well as agonist-induced β-adrenergic receptor dimerization (42), we suggest that receptor dimerization may be a primary event following agonist binding to G protein-coupled receptors. We thus amplify earlier interpretations by suggesting that the inability of CCR2BY139F to promote Gαi protein association results from the failure of the receptor to dimerize. Since signaling by G protein-coupled receptors leads to calcium mobilization within seconds, we postulate that the ligand promotes a conformational change that stabilizes the receptor dimers.

Finally, the dimerization hypothesis may also be helpful in understanding how chemokines prevent HIV-1 infection, as we conjecture that receptor dimerization prevents HIV-1 interaction with the chemokine receptor. Recent reports indicate that heterodimerization between CCR5 and its mutant CCR5Δ32 is a molecular explanation for the delayed onset of AIDS in heterozygous CCR5/CCR5Δ32 individuals (43). Our results also extend the model of cytokine receptor signaling to the chemokines, which are functionally related molecules although they use structurally unrelated receptors.

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References


