The N-terminal Extracellular Segments of the Chemokine Receptors CCR1 and CCR3 Are Determinants for MIP-1α and Eotaxin Binding, Respectively, but a Second Domain Is Essential for Efficient Receptor Activation*  

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CC1 and CCR3 are seven-transmembrane domain G protein-coupled receptors specific for members of the CC chemokine subgroup of leukocyte chemoattractants. Both have been implicated in the inflammatory response, and CCR3, through its expression on eosinophils, basophils, and Th2 lymphocytes, may be especially important in allergic inflammation. CCR1 and CCR3 are 54% identical in amino acid sequence and share some ligands but not others. In particular, macrophage inflammatory protein 1α (MIP-1α) is a ligand for CCR1 but not CCR3, and eotaxin is a ligand for CCR3 but not CCR1. To map ligand selectivity determinants and to guide rational antagonist design, we analyzed CCR1:CCR3 chimeric receptors. When expressed in mouse pre-B cells, chimeras in which the N-terminal extracellular segments were switched were both able to bind both MIP-1α and eotaxin, but in each case, binding occurred via separate sites. Nevertheless, neither MIP-1α nor eotaxin were effective agonists at either chimeric receptor in either calcium flux or chemotaxis assays. These data are consistent with a multi-site model for chemokine-chemokine receptor interaction in which one or more subsites determine chemokine selectivity, but others are needed for receptor activation. Agents that bind to the N-terminal segments of CCR1 and CCR3 may be useful in blocking receptor function.

Chemokines and their seven-transmembrane domain G protein-coupled receptors constitute a large and highly differentiated signaling system involved in multiple biologic processes, including development, hematopoiesis, angiogenesis, and regulation of specific leukocyte trafficking (1–3). Together the system is capable of supporting host defense and repair functions but may also act as an amplifier of inappropriate inflammation in diseases such as asthma. Moreover, many components of the chemokine system are exploited as pro-microbial factors (3, 4). For example, diverse chemokine receptors can be exploited as cell entry factors by HIV-1 (5).  

As the chemokine system expanded by gene replication, some functions were conserved, but new ones were added, allowing functional back-up to grow apace with functional diversification. This is illustrated nicely by the receptors CCR1 and CCR3. These receptors are more closely related in sequence to each other than to other chemokine receptors (54% amino acid identity) and have overlapping but nonidentical functional specificities (6–12). Both bind multiple chemokines, but only members of the CC subgroup of chemokines, including RANTES and MCP-3. However, each receptor also binds a selective ligand, eotaxin in the case of CCR3 and MIP-1α in the case of CCR1. Another differential feature is that CCR3 is an HIV-1 coreceptor, whereas this activity has not been found for CCR1 (13–17).  

CC1 is the only known eotaxin receptor, whereas several MIP-1α receptor subtypes have been identified (18–20). Eotaxin is a major activator of eosinophils, basophils, and Th2 lymphocytes, acting by binding to CCR3 (21–23), which has suggested that eotaxin and CCR3 are major factors regulating allergic inflammation. Consistent with this, mice rendered deficient in eotaxin by gene targeting exhibit reduced eosinophilic inflammation in response to both allergen challenge of the airway and cornea (24).  

Compared with CCR3, CCR1 appears to be expressed at higher levels on lymphocytes and monocytes and at lower levels on eosinophils (25, 26). In mice it is an important neutrophil chemotactic receptor, but this function may not be expressed in humans (26, 27). Mice lacking CCR1 have increased susceptibility to Aspergillus infection, reduced granulomatous responses to Schistosome egg challenge, and reduced pneumonitis in a pancreatitis-induced pneumonitis model (27, 28).  

Because of their roles in inflammation, identifying agents that specifically block CCR1 and CCR3 function may be therapeutically useful, and information about the ligand binding site may help to develop the most efficacious blocking agents. Also, CCR3 binding agents may be useful as anti-HIV agents. Eotaxin itself has this property, whereas RANTES and MCP-3 are much less potent (17). We have previously analyzed a series of chimeric CCR1:CCR3 receptors to map determinants of the HIV-1 coreceptor activity at CCR3 (17). Here we use chimeric receptors to map chemokine selectivity determinants for CCR1 and CCR3.

EXPERIMENTAL PROCEDURES  

Construction of Chimeric Receptors—Construction of DNA-encoding chimeric receptors was accomplished by overlap extension polymerase chain reaction using the p4 cDNA-encoding CCR1 and the clone 3 cDNA-encoding CCR3 as templates, as described previously (7, 9, 17). The sequence encoding the FLAG epitope (Eastman Kodak Co.) was inserted between the first two codons by inclusion in the 5′ oligonucleotide primer. Chimeric DNA was then ligated into the vector pcDNA3

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† The abbreviations used are: HIV, human immunodeficiency virus; MIP, macrophage inflammatory protein 1α.

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19972
proceed at room temperature for 1 h, after which 500 m
buffer adjusted to 0.5 M NaCl was added, and the cells were pelleted by
lished observations.

0.25 nM labeled ligand and varying concentrations of cold competing
of this suspension were added to duplicate Eppendorf tubes containing
every 200 ms as the relative ratio of fluorescence emitted at 510 nm
Results obtained were expressed as a chemotactic index.
using microchemotaxis chambers (Neuroprobe, Cabin John, MD) (29). We have previously analyzed HIV-1 specificity deter-
transfected cell lines in a mouse pre-B cell lymphoma cell line.
We therefore subcloned the chimeric DNA inserts into
study because vaccinia interrupts G protein signaling in these
moter in the plasmid vector pSC59, allows high levels of recep-
results denoted are from representative experiments performed on a
single clone at least 3 times.

RESULTS AND DISCUSSION

Differential Chemokine Specificities of Wild Type CCR1 and
and 200 nM unlabeled eotaxin as probes (data not shown).
versely, clone L expressing CCR3 exhibited specific binding for
eotaxin, but specific MIP-1α binding was not detected on the same cells using 0.25 nM 125I-MIP-1α and 200
2 G. Alkhatib, M. Locati, P. M. Murphy, and E. A. Berger, unpub-
and sequences

FIG. 1. Differential specificity of CCR1 and CCR3 for MIP-1α
a and eotaxin. Results in each panel correspond to the receptor indi-
cated at the top of the column in which it is found. A and B, calcium flux
ssay. Pre-B cells stably expressing CCR1 or CCR3 were stimulated with the indicated concentrations of eotaxin (open circles) or MIP-1α
(closed circles), and calcium flux was monitored in real time by Fura-2
fluorescence. The maximum of the fluorescence change observed at each
concentration is plotted on the y axis. Results are from a single clone for
each construct and are representative of 2–3 clones tested 3 times.

C and D, chemotaxis assay. Each panel gives the results of chemotaxis
assays in response to various concentrations of eotaxin (open circles) or
MIP-1α (closed circles). E and F, binding assay. Each panel gives the
results of homologous competition for 0.25 nM radioligand indicated
using increasing concentrations of the same unlabeled chemokine. B/T,
bound chemokine/total chemokine. In each panel of the figure, the
results denoted are from representative experiments performed on a
single clone at least 3 times.

2200 Ci/mmol. Cells were washed once in binding buffer (Hanks’ buff-
ered saline solution containing 1% bovine serum albumin and 0.05%
NaCl) and resuspended in the same buffer at 2 × 10⁶ cells/ml. 100 µl of
this suspension were added to duplicate Eppendorf tubes containing
0.25 nM labeled ligand and varying concentrations of cold competing
ligand in a final volume of 200 µl. Ligand binding was allowed to
proceed at room temperature for 1 h, after which 500 µl of binding buffer adjusted to 0.5 M NaCl was added, and the cells were pelleted by
centrifugation at 1.5 × 10⁶ g for 5 min. The supernatant was aspirated,
and the cell pellet was cut from the tube and counted in a gamma
counter. Nonspecific binding was typically 20–40% of the total counts.
The data were fit to a curve, and the apparent binding affinity and
receptor density were estimated using the program LIGAND.

RESULTS AND DISCUSSION

Differential Chemokine Specificities of Wild Type CCR1 and
CCR3—We have previously analyzed HIV-1 specificity deter-
motors for CCR3 using a panel of CCR1:CCR3 chimeric re-
tors transiently expressed in NIH 3T3 cells. This system,
which depends on vaccinia virus activation of a vaccinia pro-
moto in the plasmid vector pSC59, allows high levels of recep-
tor expression; however it could not be used for the present
study because vaccinia interrupts G protein signaling in these
cells. We therefore subcloned the chimeric DNA inserts into
the mammalian expression vector pcDNA3 and cloned stably
transfected cell lines in a mouse pre-B cell lymphoma cell line.

Untransfected cells did not exhibit specific binding of MIP-1α
or eotaxin at the concentrations employed. However, very small
calcium fluxes were observed on untransfected cells in response to
MIP-1α but only at concentrations of 300 nM and above (data not shown).
Conistent with previous reports, after transfection with the CCR1 expression plasmid, approximately 50% of
cloned tested responded to MIP-1α at low nanomolar concen-
trations in a calcium flux assay, but none responded to eotaxin.
Likewise, after transfection with the CCR3 expression plasmid,
approximately 40% of clones tested responded to eotaxin in the
calcium flux assay, but none responded to MIP-1α. Clones that
gave the most robust responses were selected for further char-
acterization (Fig. 1). Clone 1 expressing CCR1 exhibited speci-
fic binding for MIP-1α (Fig. 1F), but specific eotaxin binding
was not detected on the same cells using 0.25 nM 125I-eotaxin

2 G. Alkhatib, M. Locati, P. M. Murphy, and E. A. Berger, unpub-
lished observations.

and 200 nM unlabeled eotaxin as probes (data not shown).
versely, clone L expressing CCR3 exhibited specific binding for
eotaxin (Fig. 1E), but specific MIP-1α binding was not detected on the same cells using 0.25 nM 125I-MIP-1α and 200
nM unlabeled MIP-1α as probes (data not shown). Scatchard analysis of competition binding data revealed that clone L
expressing CCR3 expressed ~2000 eotaxin binding sites/cell with an apparent Kᵅ = 0.5–1.0 nM (Table 1). The EC₅₀ for
calcium release by eotaxin in these cells was 1–5 nM (Fig. 1A and Table 1). Eotaxin binding was insensitive to competition with excess MIP-1α, and MIP-1α did not antagonize eotaxin
induction of calcium release (data not shown). Receptor param-
eters for clone 1 expressing CCR1 were similar: ~2200 MIP-1α
binding sites/cell, with an apparent Kᵅ = 1–5 nM, and an EC₅₀ for
calcium release by MIP-1α of 1–5 nM (Table 1). Also, MIP-1α
binding to clone 1 was insensitive to competition with excess
eotaxin, and eotaxin did not antagonize MIP-1α induction of
calcium release (data not shown). These parameters are con-
istent with those previously reported for wild type CCR1 and
CCR3 expressed in other cell types (6, 11, 12).

Clone L cells expressing CCR3 also exhibited chemotaxis to
eotaxin, with the maximal response observed at a concentra-
tion of 100 nM (Fig. 1C). No background chemotaxis was
observed with eotaxin using untransfected cells, even at concentrations of 1 μM. In contrast, MIP-1α was a poor chemoattractant, eliciting a chemotactic response only when employed at 1 μM, which represented a background response on these cells (Fig. 1C and data not shown). Clone 1 cells expressing CCR1 were unable to chemotax in response to eotaxin, as may have been inferred by the calcium release data in panel B, but surprisingly, MIP-1α was also a poor attractant, eliciting only background chemotactic activity when tested at 1 μM (Fig. 1D). Similarly, chemotaxis results were obtained when the same constructs were tested in transiently transfected L1.2 cells (data not shown).

It is interesting that two similar receptors expressed in the same cellular background can exhibit potent efficacy in mobilizing intracellular calcium to key ligands, yet have such different chemotactic profiles to the same ligands. Similar but converse findings have been observed for CCR2B, where chemotaxis to MCP-1 was observed in the absence of a calcium flux, suggesting that the two events can occur independently of each other (30).

Staining of both clones with the anti-FLAG monoclonal antibody M5 revealed a small 2-fold shift relative to staining of untransfected cells, consistent with the low levels of expression determined by Scatchard analysis of the binding data.

Mapping of Ligand Specificity Determinants to the N-terminal Extracellular Segments of CCR1 and CCR3—Having established the receptor parameters for wild type CCR1 and CCR3 in our system, we next tested the importance of the N-terminal segments of each receptor in determining differential ligand selectivity. We focused on this region because the corresponding region has been shown to be a ligand selectivity determinant for several other chemokine receptors, including CXCR1, CXCR2, Duffy and CCR2 (31–37).

One chimera contained amino acids 1–32 from CCR1 joined to amino acids 33–355 of CCR3 and was named CHI1. The reciprocal chimera contained amino acids 1–32 of CCR3 fused to amino acids 33–356 of CCR1 and was named CHI2. The junctions were based on the hydropathy plot of the receptors. When expressed in pre-B cells, both chimeras exhibited chimeric ligand recognition, specifically binding both 125I-MIP-1α and 125I-eotaxin (Fig. 2).

Scatchard analysis of competition binding data revealed that clone A6 for CHI1 coexpressed both eotaxin and MIP-1α binding sites (apparent Ki 5–10 nM and 10–50 nM, respectively, and 6,000–10,000 sites/cell for each). Likewise, Scatchard analysis of competition binding data revealed that clone 5.5 for CHI2 coexpressed both eotaxin and MIP-1α binding sites (apparent Ki 0.5–1.0 nM and 50–75 nM, respectively, and 6,000–10,000 sites/cell for each).

A role for the N-terminal segment of CCR1 as a MIP-1α binding selectivity determinant is supported by the gain of MIP-1α binding in CHI1 relative to wild type CCR3 and the 50-fold reduction in apparent binding affinity for MIP-1α in CHI2 relative to wild type CCR1. A role for the N-terminal segment of CCR3 as an eotaxin binding selectivity determinant is supported by the gain of eotaxin binding in CHI2 relative to

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**TABLE 1**

| Receptor   | Binding K<sub>i</sub> | [Ca<sup>2+</sup>] flux EC<sub>50</sub> |
|------------|----------------------|----------------------------------|
|            | MIP-1α | Eotaxin | MIP-1α | Eotaxin |
| CCR3       | ND     | 6.7 ± 2.3 | ND     | 1–5    |
| CCR1       | 0.7 ± 0.4 | ND     | 1–5    | ND     |
| CHI-1      | 26.3 ± 5.2 | 17.5 ± 1.6 | ND     | ND     |
| CHI-2      | 4.6 ± 0.1 | 15.2 ± 3.8 | ND     | ND     |
| CHI-3      | 5.1 ± 1.2 | 21.7 ± 9.4 | 1–5    | ND     |

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**Fig. 2.** Importance of the N-terminal extracellular segment of CCR1 and CCR3 for chemokine specificity. Results in each panel correspond to the receptor construct indicated at the top of the column in which it is found. Black indicates CCR1 sequence; white indicates CCR3 sequence. A–D, ligand binding assay. Each panel gives the results of homologous competition for 0.25 nM radioligand shown in each box using increasing concentrations of the same unlabeled chemokine. Results are from representative experiments performed on a single clone, tested 2–3 times. B/T, bound chemokine/total chemokine.
wild type CCR1 and the −10-fold reduction in apparent binding affinity for eotaxin in CHI1 relative to wild type CCR3. That other receptor domains are also involved in ligand binding selectivity is apparent from the preserved ability of CHI1 to bind eotaxin and CHI2 to bind MIP-1α, albeit at reduced affinity.

Independence of MIP-1α and Eotaxin Binding Sites in CCR1: CCR3 Chimeric Receptors—Heterologous competition binding was carried out to examine the relationship of the MIP-1α and eotaxin binding sites on CHI1 and CHI2. Neither chemokine could compete for binding to the site labeled by the other on either chimera, even when 1 μM heterologous chemokine was used (data not shown), suggesting that the sites are independent, as they are on wild type CCR1 and wild type CCR3. Independence of binding sites has also been reported for interleukin-8 and GROα on CXCR1:CXCR2 chimeric receptors and for MCP-1 and MIP-1α on chimeric receptors (33–36). In the case of CCR2, a pseudo-tethered N-terminal domain binds MCP-1 with affinity similar to the full-length wild type receptor, suggesting that the bulk of the binding energy between CCR2 and MCP-1 is conferred by interactions occurring solely between MCP-1 and this domain (36). Also, there is direct structural evidence from spectroscopy studies for binding of interleukin-8 to the N-terminal segment of CXCR1 (38). Ligand selectivity determinants have been mapped to the second extracellular loop of CCR5 using CCR2:CCR5 chimeras and to the third extracellular loop of CCR1 using CCR1:CCR2 chimeras; however, direct binding sites have not been identified yet (39, 40). Thus, in some cases chemokines may bind to receptors at a single extracellular domain, and this domain could differ for different chemokines binding to the same receptor. However, in other cases, ligands appear to share sites on the same receptor.

It is important to point out that, in contrast to our results with CCR1:CCR3 chimeras, MIP-1α selectivity did not map to the N-terminal segment of CCR1 in CCR1:CXCR2 or CCR1: CXCR4 chimeras reported previously (33, 35). Our data suggest that a revised interpretation of these negative results may be warranted: that the CCR1 N-terminal segment complements other domains to determine MIP-1α recognition specificity. From this perspective, CCR3 contains complimentary domains, whereas CXCR2 and CCR2 do not.

Requirement for Other Receptor Domains for Receptor Activation—With several exceptions, there is a fairly good correlation between the rank order of chemokine binding affinity for individual receptors and among different receptors versus the rank orders when the same chemokines are evaluated as agonists. Creation of chimeric receptors allows one to test whether chemokine binding specificity determinants and chemokine receptor activation determinants map to the same domains.

Unlike wild type CCR1 and wild type CCR3, we did not observe activation of either CHI1- or CHI2-expressing stable cell lines in calcium flux and chemotaxis assays when stimulated with either MIP-1α or eotaxin, even at concentrations 10−100-fold greater than the apparent binding constant. The cells were capable of signaling as shown by robust calcium flux responses to the CXC chemokine SDF-1 via an endogenous pre-B cell receptor that is probably the chemokine receptor CXCR4 (not shown; Refs. 41 and 42). Moreover, the results could not be explained by low receptor expression, since levels of chimeric receptors were actually 3-fold higher than wild type receptor levels in the clones tested. Nor could they be explained completely by reduced ligand binding affinity, since eotaxin binding affinity for CHI2 and wild type CCR3 were equivalent. In the case of MIP-1α, affinity for CHI1 and CHI2 was substantially reduced (−10-fold) compared with wild type CCR1. However, a third chimeric receptor named CHI3, in which the third extracellular loop of CCR1 was replaced with that of CCR3, was expressed at levels similar to CHI1 and CHI2, exhibited an affinity for MIP-1α only 5-fold higher than values for CHI1 and CHI2, and supported MIP-1α signaling at a potency and efficacy equivalent to wild type CCR1 (EC50 = 1−5 nM for calcium release, Fig. 3). Cells expressing CHI3 were able to bind eotaxin with an apparent affinity −3-fold lower than for wild type CCR3 (−20 nM). However, no response to eotaxin was observed at concentrations up to 200 nM. The reciprocal of CHI3 was not informative in fluorescence-activated cell sorter, ligand binding, or calcium flux assays (data not shown). Note that the present CHI3 is a different structure from a construct named CHI3 in a previous paper (17).

Similar results were obtained when the same constructs were transiently expressed in L1.2 pre-B cells, with one exception. CHI1-transfected cells responded chemotactically to eotaxin, although the efficacy was typically −25% that of wild type CCR3 in the same system, despite equivalent levels of expression as determined by the monoclonal antibody 7B11 (data not shown). Thus the results from the stably and transiently transfected cells with respect to chemotaxis are qualitatively similar.
Two-site Model for Chemokine Receptor Activation

Conclusions—The results of the present study are consistent with a multi-site model for chemokine-chemokine receptor interaction in which one or more subsites determine chemokine selectivity, but others are needed for receptor triggering. In this model, ligand is envisioned to bind first via a docking domain, which may be the N-terminal segment for eotaxin and MIP-1α in the case of CCR3 and CCR1, respectively, and then to be presented to a second activation site on the receptor. The first well documented example in support of this model was the C5a receptor, which binds a peptide chemoattractant similar in size to the chemokines (43). Since then, additional examples have been reported based on the study of chimeric CCR1: CXC2R, CCR2:CCR5, and CXCR1:CCXCR2 receptors (33–36).

Analysis of chimeric receptors only allows statements about selectivity determinants and does not give direct information about the location of ligand binding sites or sites important in receptor activation. In the case of CCR2 and CCR1, the importance of the N-terminal segment in binding MCP-1 and IL-8, respectively, which had been suggested from chimeric receptor studies, was later validated by more direct experimental approaches (36, 38). In the case of CCR1 and CCR3, direct identification of functional sites has not yet been accomplished. Nevertheless, since the N-terminal segments of CCR1 and CCR3 appear to be important ligand binding selectivity determinants, agents that bind there could perturb receptor recognition of MIP-1α and eotaxin, respectively, and act as effective blocking agents, either directly or allosterically. Point mutagenesis of these regions should enable a more detailed understanding of the functional sites on CCR1 and CCR3 and may further assist the development of specific antagonists.

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