The Second Extracellular Loop of CCR5 Is the Major Determinant of Ligand Specificity

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The chemokine receptor CCR5 binds macrophage inflammatory protein (MIP)-1α, MIP-1β, and regulated on activation, normal T-cell expressed and secreted (RANTES), and constitutes the major co-receptor allowing infection of CD4+ T lymphocytes, macrophages, and microglial cells by macrophage-tropic strains of human and simian immunodeficiency virus. CCR5 is most closely related to CCR2b, another chemokine receptor that responds to monocyte chemotactic protein (MCP)-1, MCP-2, MCP-3, and MCP-4. We have investigated by mutagenesis the regions of CCR5 and CCR2b involved in the specificity of binding and functional response to their respective ligands. We demonstrate that the key region of CCR5 involved in its specific interaction with MIP-1α, MIP-1β, and RANTES, and its subsequent activation, lies within the second extracellular loop (and possibly the adjacent transmembrane segments). Conversely, the N-terminal domain of CCR2b is responsible for the high affinity binding of MCP-1, but is not sufficient to confer activation of the intracellular cascades. Extracellular loops of the receptor, among which the second loop plays a prominent role, are necessary to achieve efficient signaling of the receptor. These data complement our previous mapping of CCR5 domains functionally involved in the fusion process with the human immunodeficiency virus envelope, and will help in the development of agents able to interfere with the early steps of viral infection.

Chemokines play fundamental roles in the physiology of acute and chronic inflammatory processes, as well as in dysregulations of these processes, by attracting and stimulating specific subsets of leukocytes (1, 2). Chemokines are divided into two main families (CXC- and CC-chemokines) depending on the relative position of the first two conserved cysteines. Their actions are mediated by subfamilies of G protein-coupled receptors. To date, four functional receptors responding to CXC-chemokines (CXCR1 to 4) and eight receptors responding to CC-chemokines (CCR1 to 8) have been identified in the human. Chemokine receptors were also identified recently as the long-searched for HIV1 co-receptors (3–5). CXCR4 was first shown to constitute an accessory factor allowing infection by T-tropic HIV-1 strains (6). CCR5 (7) was further demonstrated to be necessary for infection by M-tropic viruses (8–12), following the identification of MIP-1α, MIP-1β, and RANTES as major HIV suppressive factors (13). A mutant allele of CCR5 strongly protects homozygotes against HIV infection and confers partial resistance and slowed disease progression to heterozygotes (14–16), demonstrating the non-redundant role of CCR5 as HIV co-receptor. Other chemokine receptors, such as CCR3 and CCR2b can also act as co-receptors for some HIV-1 strains, in addition to CCR5 and CXCR4 (12).

Chemokines and chemokine analogs are able to inhibit HIV-1 infection in vitro, and are believed to be partially protective in vivo (13, 17–19). To understand this interaction at the molecular level, and to design more active molecules, it is critical to determine what regions of CCR5 are responsible for the specificity of chemokine binding and co-receptor activity. Relatively little is known concerning the structure-function relationships of CC-chemokine receptors and their ligands, in general. Monteclaro and Charo (20) reported that the amino terminus of CCR2b is necessary for MCP-1 binding, with the extracellular loops being important for receptor activation and signaling. They have also shown that the third extracellular loop, but not the amino terminus of CCR1, is required for ligand binding and signal transduction (20). We have shown previously that the NH2 terminus and the first extracellular loop of CCR5 are responsible for the specificity of interaction with M-tropic HIV-1 strains (21).

In the present study, we analyzed the regions of CCR5 and CCR2b that determine the specificity of binding and functional response to their respective ligands, using a set of CCR5/CCR2b chimeric receptors. Indeed, although the two receptors are closely related structurally, their ligand specificity is mutually exclusive: CCR2b responds to MCP-1, MCP-2, MCP-3, and MCP-4 (22, 23),2 while CCR5 is activated by MIP-1α, MIP-1β, and RANTES (7, 24, 25). We found that the second extracellular loop of CCR5 is critical for high affinity binding of MIP-1α, MIP-1β, and RANTES, and for the functional response to these chemokines. By contrast, the amino-terminal domain

1 The abbreviations used are: HIV, human immunodeficiency virus; CHO, Chinese hamster ovary; CCR, CC chemokine receptor; GRO, growth-related gene product; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; RANTES, regulated on activation, normal T-cell expressed and secreted.
2 G. LaRosa, unpublished observations.
of CCR2b is responsible for the high affinity binding of MCP-1 and MCP-3, but other regions, including the second extracellular loop of the receptor, are necessary for efficient signal transduction.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human chemokines, including MCP-1, MCP-3, MIP-1α, MIP-1β, and RANTES, were obtained from R & D Systems (London, United Kingdom). 125I-MCP-1, 125I-MIP-1α, and 125I-MIP-1β (specific activity, 2200 Ci/mmol) were obtained from New England Nuclear (Cambridge, MA). Chemokines obtained from R & D Systems were reported by the supplier as >97% pure on SDS-polyacrylamide gel electrophoresis and biologically active on a biosassay specific for each ligand. They were shown locally to be active at adequate concentrations on at least one recombinant chemokine receptor expressed in CHO-K1 cells. Lyophilized chemokines were dissolved as 100 μg/ml or 1 mg/ml solutions in sterile phosphate-buffered saline and stored at −20 °C in aliquots. They were diluted to the working concentration immediately before use.

CCR2b/CCR5 Chimeric Constructs—The CCR5, CCR2b, and CCR5/CCR2b chimeric receptors were cloned between the BamHI and XbaI sites of pcDNA3, as described previously (21). Briefly, an AflII restriction site was introduced by site-directed mutagenesis in a region corresponding to the end of the first transmembrane region of each receptor. The various chimeras were generated by transferring restriction fragments flanked by the common BamHI, AflII, ClaI, EcoRI, and XbaI sites between the CCR5 and CCR2b constructs. After checking the constructs by sequencing, they were all transferred into a bicistronic vector (26) under the control of the elongation factor 1α promoter.

Expression in Cell Lines—CHO-K1 cells were cultured using Ham’s F-12 medium supplemented with 10% fetal calf serum (Life Technologies, Inc.). The CCR5, CCR2b, and CCR5/CCR2b chimeric receptors were cloned between the first extracellular loop of CCR5 and the second extracellular loop of CCR5 (22). 125I, 125I-MIP-1α, 125I-MIP-1β, and 125I-RANTES were used at 30 nM concentration, were potent activators of CCR5, MCP-1 and MCP-3 had no effect on the metabolic activity at the same concentration. In contrast, MCP-1 and MCP-3 activated CCR2b while MIP-1α, MIP-1β, and RANTES had no effect on CCR2b.

Functional Response of Wild-type CCR5 and CCR2b—The cell lines expressing CCR5 and CCR2b were also tested for their biological response to various chemokines, by using a microphysiometer. Fig. 2 (top panels) shows that MIP-1α, MIP-1β, and RANTES, used at a 30 nM concentration, were potent activators of CCR5. MCP-1 and MCP-3 had no effect on CCR2b and vice versa. Using CHO-K1 cells stably expressing these constructs in binding assays, we could determine whether a single (or combinations of) extracellular domain(s) of CCR5 was able to confer the ability to bind the CCR5-specific ligand MIP-1α. A similar analysis was made for CCR2b domains and the CCR2b-specific ligand MCP-1. The chimeric receptors were generated using restriction sites naturally shared by CCR5 and CCR2b cDNAs, such as ClaI, XbaI, and EcoRI, or introduced by silent mutagenesis in both sequences (AflII site) (Fig. 1A).

In a first series, we tested a set of chimeras scanning the extracellular domains of CCR5 and CCR2b for their involvement in the MIP-1α or MCP-1 binding specificity. Starting from the structure of CCR5 (5555), the constructs included an increasing number of CCR2b domains (2555, 2255, 2225, and 2222), and were compared with untransfected CHO-K1 cells. As shown in Fig. 3, B and D, the replacement of the NH2-terminal domain of CCR5 by the corresponding region of CCR2b (2255), and the replacement of the NH2 terminus and the first extracellular loop (2225) did not affect the ability of the chimeric receptors to bind MIP-1α, although this affinity was slightly impaired (Table I). The additional replacement of the second extracellular loop of CCR2b (2225) completely abolished specific MIP-1α binding. By contrast, it was found that the replacement of the NH2-terminal segment of CCR5 by the cognate domain of CCR2b (2555) was sufficient to confer MCP-1 binding (Fig. 3, C and E). Additional replacements of
the first and second extracellular loops (2255 and 2225) did not modify the binding properties significantly. Once again, chimeric receptors had a somehow lower affinity for MCP-1 than wild-type CCR2b (Table I).

From this first set of data, it appeared therefore that the NH₂-terminal domain of CCR2b was essential for high affinity MCP-1 binding, while the second extracellular loop of CCR5 was the main determinant for its MIP-1α binding properties.

To confirm these observations, we tested a second set of constructs shuffling the CCR5 and CCR2b domains differently. As shown in Fig. 4B, the replacement of the second loop of CCR5 by the second loop of CCR2b (5525) was able to abrogate completely MIP-1α binding. The 5255 chimera, with a similar replacement of the first extracellular loop, retained MIP-1α binding activity. The role of the second extracellular loop was further confirmed by the properties of the 2252 chimera, where this single CCR5 loop in a CCR2b background was able to confer an affinity for MIP-1α comparable to that of wild-type CCR5. The NH₂ terminus of CCR5 alone (5222) was totally ineffective.

MCP-1 binding on the same chimeras confirmed the predominant role of the NH₂-terminal segment of CCR2b (Fig. 4C). The replacement of the NH₂-terminal domain of CCR2b was essential for high affinity MCP-1 binding, while the second extracellular loop of CCR5 was the main determinant for its MIP-1α binding properties. To confirm these observations, we tested a second set of constructs shuffling the CCR5 and CCR2b domains differently. As shown in Fig. 4B, the replacement of the second loop of CCR5 by the second loop of CCR2b (5525) was able to abrogate completely MIP-1α binding. The 5255 chimera, with a similar replacement of the first extracellular loop, retained MIP-1α binding activity. The role of the second extracellular loop was further confirmed by the properties of the 2252 chimera, where this single CCR5 loop in a CCR2b background was able to confer an affinity for MIP-1α comparable to that of wild-type CCR5. The NH₂ terminus of CCR5 alone (5222) was totally ineffective.

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Biological Activity of CCR5/CCR2b Chimeric Constructs—Binding assays allowed us to demonstrate unambiguously the respective role of the various domains of CCR5 and CCR2b in the high affinity binding specificity of the receptors toward MIP-1α or MCP-1. Since binding properties do not necessarily correlate with the ability of receptors to activate intracellular cascades, we tested the same set of constructs, as well as some additional chimeras for their signal transduction properties. The microphysiometer was used for this purpose. All cell lines were stimulated in triplicate by a 30 nM concentration of the three natural ligands of CCR5 (MIP-1α, MIP-1β, and RANTES) and two of the natural ligands of CCR2b (MCP-1 and MCP-3). As shown in Fig. 5A, the ability of the 2555, 2255, and 2225 chimeras to respond functionally to MIP-1α (but also to MIP-1β and RANTES, not shown) was in perfect accordance with the MIP-1α binding data (Fig. 3, B and C), suggesting that the second extracellular loop is responsible for the high affinity binding of CCR5 agonists, but also for the ability of the receptor to respond functionally to these agonists. These conclusions were verified on an extended set of chimeras (Fig. 5B). Once again, the functional data confirmed that all chimeras binding second extracellular loop of CCR5 (not shown).

**Fig. 3.** MIP-1α and MCP-1 binding to CCR5/CCR2b chimeric receptors. A, schematic representation of a set of chimeric receptors scanning the extracellular domains for their involvement in ligand specificity. Domains originating from CCR5 and CCR2b are, respectively, represented in black and grey. The location of the A/III, C/II, and EcoRI restriction sites used in domain exchange is represented, as well as the putative glycosylation sites. B and D, competition binding curves performed on cells expressing CCR5, CCR2b, or the chimeric receptors, and untransfected CHO-K1 cells, using 125I-MIP-1α as radioligand, and unlabeled MIP-1α as competitor. C and E, competition binding curves performed on cells expressing CCR5, CCR2b, or the chimeric receptors, and untransfected CHO-K1 cells, using 125I-MCP-1 as radioligand, and unlabeled MCP-1 as competitor. Results are expressed as the ratio of bound tracer to total input tracer.

**Fig. 4.** Confirmation of the domains involved in ligand selectivity. A, a second set of chimeras were tested for their ability to bind 125I-MIP-1α and 125I-MCP-1 to confirm the role of the various extracellular domains. The chimeras are schematically represented, with CCR5 and CCR2b domains in black and grey, respectively. B, competition binding curves performed on cell lines expressing the chimeric receptors, using 125I-MIP-1α as radioligand and unlabeled MIP-1α as competitor. C, competition binding curves using 125I-MCP-1 as radioligand and unlabeled MCP-1 as competitor. Results are expressed as the ratio of bound tracer to total input tracer.
MIP-1α (Fig. 4A) were able to stimulate cell metabolism in response to this agonist, and to MIP-1β and RANTES as well (not shown). Functional response to CCR5 agonists was entirely correlated with the presence of the second extracellular loop of CCR5. All chimeras containing this region supported signal transduction induced by MIP-1α. In contrast, all chimeras lacking this domain failed to support signal transduction.

Regarding the analysis of extracellular domains of CCR2b involved in signal transduction, Fig. 6A shows that the correlation with MCP-1 binding activity was not as strong as that seen for CCR5. Indeed, although the 2555 and 2255 chimeras were able to bind MCP-1 efficiently (Fig. 3C), the functional response of the cells expressing these constructs was weak as compared with wild-type CCR2b. The additional replacement of the second extracellular loop (2225) was necessary to confer a functional response comparable to that of CCR2b. These first results suggested therefore that the NH₂-terminal segment of CCR2b is able to bind MCP-1 with high affinity, but that other domains, such as the second extracellular loop, are required for efficient signal transduction. Once again, these conclusions were verified and extended by testing the functional response of additional chimeras to MCP-1 (Fig. 6B) and MCP-3 (not shown). The first extracellular loop of CCR2b, alone (5255) or associated with the third loop (5252) failed to support MCP-1-induced signal transduction. The second extracellular loop, together with the NH₂-terminus of CCR2b (2525) was among the most effective chimeras in terms of functional response, confirming the role of the two loops. Nevertheless, the second loop is not absolutely necessary, since the 2252 construct lacking the second extracellular loop of CCR2b responds as well as the 2525 chimera. More interestingly, chimeras such as 5525, and to a lower extent 5222 and 5225 that do not include the CCR2b NH₂-terminus and did not show appreciable binding of MCP-1 in the competitive binding assays (Fig. 4C), were able to respond functionally to MCP-1 and MCP-3. Altogether, the functional data indicate that the NH₂-terminal domain of CCR2b, which is necessary for MCP-1 high affinity binding is not sufficient to confer signal transduction. The second extracellular loop of CCR2b is by itself the most efficient domain conferring signal transduction capability, although it is dispensable when all other domains or CCR2b are present (2252). Signal transduction can occur in the absence of the high affinity binding site of the NH₂-terminus.

As already mentioned, all chimeras were tested for their functional response to the CCR5 agonists MIP-1α, MIP-1β, RANTES, and to the CCR2b agonists MCP-1 and MCP-3, to determine whether the same regions of the receptors mediate the interaction with the various ligands. A selected set of data illustrating these experiments is represented in Fig. 7. As shown in panel A, MIP-1α, MIP-1β, and RANTES, used at a 30 nM concentration, promote a similar increase in metabolic activity on cells expressing wild-type CCR5. The three chemokines are all inactive on chimeric receptor 5555, when the second extracellular loop is swapped with that of CCR2b. The reverse chimeric receptor 2252, including the second extracellular loop of CCR5 only, responds similarly to the three chemokines. Fig. 5B shows that MCP-1 and MCP-3 at a 30 nM concentration both activate wild-type CCR2b-transfected cells, MCP-1 being more potent than MCP-3, in accordance with their different affinity, as deduced from the binding assays. Chimera 5252 exhibits almost no response to either chemokines, while chimera 2525 is activated by both. The chimeric receptor 2252, that is activated by MIP-1α, MIP-1β, and RANTES, responds also to MCP-1 and MCP-3. For all other chimeras tested, the response to MIP-1β and RANTES was similar to that of MIP-1α, and the response to MCP-3 was concordant, although frequently weaker, to that of MCP-1.

**DISCUSSION**

CCR5 constitutes the main functional co-receptor for M-tropic HIV-1 strains, since homozygote carriers for the mutant allele of the CCR5 gene are strongly resistant to infection by the virus, both in vitro and in vivo (14–16). Direct interaction between the envelope protein of HIV-1, CD4, and CCR5 has been demonstrated (29, 30). It has also been widely documented that chemokines and chemokine analogs endowed with
antagonistic properties are able to reduce the effectiveness of HIV-1 infection in vitro (13, 18, 19), and presumably in vivo as well (17). Blocking the interaction of the virus with CCR5 by chemokines or analogs, monoclonal antibodies, or small chemicals could therefore constitute a major therapeutic implementation in the anti-HIV arsenal. The precise knowledge of the molecular interaction between CCR5 and chemokines on one side, and CCR5, gp120, and CD4 on the other side would help in designing molecules able to interfere efficiently with the early steps of viral entry.

We have previously investigated the role of the various regions of CCR5 in the ability of the co-receptor to interact with gp120 from various strains of HIV-1 (and possibly with CD4) and initiate the fusion process (21). It was concluded that the first part of the NH2-terminal domain, as well as the first extracellular loop constitute the main regions conferring co-receptor properties to CCR5. This study, as well as similar studies made with simian immunodeficiency virus (31), showed that different viruses and strains, although using the same co-receptor (CCR5), are not dependent upon the same regions of the molecule for their specific interaction. We have now started to investigate the regions of CCR5 involved in chemokine recognition and subsequent functional response, using our panel of chimeric receptors. Our approach was based on the construction of chimeras between CCR5 and CCR2b. CCR2b is the chemokine receptor that presents the highest similarity with CCR5 (78% identity). By contrast, the ligand specificities of CCR2b and CCR5 are mutually exclusive so far, since CCR5 binds MIP-1α, MIP-1β, and RANTES, but no other known CC-chemokine (7, 24, 25), while CCR2b binds MCP-1, MCP-2, MCP-3, and MCP-4 (22, 23), but not MIP-1α, MIP-1β, and RANTES. The ligand specificity is expected to be mediated partially by the extracellular domains of the receptor that share only 45% amino acid identity, while transmembrane segments and cytoplasmic loops are highly conserved (95% identity).

We have first characterized the binding properties of CHO-K1 cell lines expressing CCR5 and CCR2b. We also showed that activation of CCR5-expressing CHO-K1 cells by MIP-α, MIP-1β, and RANTES can efficiently be monitored by a microphysiometer that measures the modifications of cell metabolism resulting from the activation of intracellular cascades (32). Similarly, stimulation of CCR2b-expressing cells by MCP-1 and MCP-3 can be monitored by this method, with a pharmacology identical to that reported previously using calcium mobilization (22, 23).

Our results demonstrate that the closely related CCR5 and CCR2b receptors interact differently with their respective ligands. For CCR5, the central role of the second extracellular loop of the receptor is obvious for both high affinity binding and activation of the receptor: the second loop of CCR5 in a CCR2b background (chimera 2252) supported MIP-1α binding and signal transduction, while the replacement of this single domain in CCR5 (chimera 5525) abolishes both. All three ligands active on CCR5 (MIP-1α, MIP-1β, and RANTES) interact apparently in a similar way with the receptor. The prominent role of the second extracellular role of CCR5 in ligand specificity is compatible with the high divergence between CCR2b and CCR5 in this region: 16 out of 26 residues are different, including an extra cysteine residue not present in any of the chemotaxtractant receptors except CCR4. This cysteine may be involved in disulphide bridging with the first extracellular loop or NH2-terminus, which could result in very different three-dimensional
structures between the two related receptors. Conserved resi-
dues include three sequential charged amino acids, and a
highly conserved cysteine believed to form a disulfide with the
first extracellular loop in all G protein-coupled receptors.

Concerning CCR2b, the amino-terminal extracellular do-
main of the receptor plays a fundamental and non-redundant
role in the high affinity binding of CC-chemokines active on
this receptor. Indeed, this single CCR2B domain in a CCR5
background was sufficient to confer MCP-1 binding. The high
sequence divergence between the NH$_2$-terminal domains of
CCR2 and CCR5 can easily account for their role in ligand
specificity. Functional data show, however, that the high affin-
ity binding site is both not sufficient and not absolutely neces-
sary to promote chemokine-mediated activation of the receptor
and of intracellular cascades. A chimera containing the CCR2b
NH$_2$ terminus (2555) binds MCP-1 but does not signal effi-
cently, while a chimera devoid of the CCR2b NH$_2$ terminus
(5525) signals in response to MCP-1 but very little binding can
be detected. These results are compatible with those reported
earlier by Montecarlo and Charo (20) on the basis of CCR2b/
CCR1 chimeras. These authors concluded similarly that the
NH$_2$ terminus of CCR2b is necessary for high affinity binding,
but that low affinity interaction with one or more extracellular
loops is necessary to achieve receptor activation and signaling.

From our data it appears that the second extracellular loop, as
well as a combination of the first and third loops, can fulfill this
function.

Our data, as well as previous structure-function studies per-
formed on other chemokine receptors, therefore support the
concept that, despite the obvious similarity in the receptor
primary structures, as well as in the three-dimensional struc-
ture of the ligands, the way chemokines interact with their
respective receptors can vary widely from one receptor to an-
other. The NH$_2$-terminal domain has been involved in ligand
specificity for other chemokine receptors of the CC and CXC
families. The NH$_2$ terminus of the interleukin-8 receptors
(CXCR1 and CXCR2) are major determinants of ligand speci-
city (33–36). The amino terminus of DARC (Duffy antigen), an
apparently non-functional receptor binding both CC and CXC
chemokines, plays a critical role in ligand binding (37). By
contrast, ligand specificity of CCR1 was reported to be medi-
ated essentially by the third extracellular loop of the receptor
(20).

The different regions involved for each receptor in ligand
binding can probably account for the diverse selectivities ob-
served among chemokine receptors. As an example, the fact
that CCR5 binds MIP-1$\alpha$, MIP-1$\beta$, and RANTES, while CCR1
binds MIP-1$\alpha$ and RANTES but not MIP-1$\beta$ is comprehensible
considering that the dominant interaction of the ligands with
the receptors are, respectively, within the second and third
extracellular loops. In our set of experiments, the clear segre-
gation of the regions, respectively, involved in high affinity
binding of MIP-1$\alpha$ and MCP-1 results in the existence of chimeric
receptors (2555, 2255) that are able to bind both CCR5 and
CCR2b ligands efficiently. It should be stressed that chimeric
receptor-based studies, such as ours, can only discrimi-
nate domains important for ligand selectivity. Conserved re-
gions that would be required for ligand binding and/or func-
tional response to the ligands could easily be overlooked.
Additional mutagenesis studies on both the receptors and li-
gands will be necessary to fully understand how chemokine
receptors interact with their respective receptor(s), and how
binding results in receptor activation and signal transduction.

From the present and previous data (21), it is clear that the
regions of CCR5 involved in chemokine ligand specificity, and
in the specificity of co-factor usage for various HIV-1 strains are
not identical. Chemokine selectivity is mediated by the second
extracellular loop, while co-factor usage is dependent on the N
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dissociation in some chimeras between viral co-receptor activity
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