CC Chemokine Receptor 5-Mediated Signaling and HIV-1 Co-receptor Activity Share Common Structural Determinants

CRITICAL RESIDUES IN THE THIRD EXTRACELLULAR LOOP SUPPORT HIV-1 FUSION*

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There is a close correspondence between the ability of RANTES and macrophage inflammatory proteins 1α and 1β to activate CC chemokine receptor 5 (CCR5) and the ability to inhibit CCR5-dependent membrane fusion mediated by the envelope glycoprotein of human immunodeficiency virus (HIV), type 1. This finding suggests that some of the structural determinants for CC chemokine/CCR5 interactions and CCR5 HIV-1 fusion co-receptor activity may be shared. Recent studies using human CCR5/CCR2B chimeras have suggested that the determinants of CCR5 co-receptor activity are complex and may involve multiple extracellular domains and that viral co-receptor activity is dissociable from ligand-dependent signaling responses. However, conclusive evidence demonstrating an important role for the second and third extracellular regions of human CCR5 is lacking. Furthermore, to determine whether the determinants for CCR5 co-receptor activity overlap with those required for agonist activity, studies that compare the chemokine specificity for inhibition of envelope-mediated cell fusion and the agonist profile of chimeric receptors are necessary. In the present report, using a series of CCR5/CCR2B chimeras we ascribe an important role for the second and third extracellular loop of CCR5 in supporting the co-receptor activity of CCR5. We also provide evidence that the intracytoplasmic tail of CCR5 does not play an important role in supporting HIV-1 entry. The hypothesis that the structural determinants for CC chemokine/CCR5 interactions and CCR5 HIV-1 fusion co-receptor activity may be shared was confirmed by two novel observations: first, the fusion activity supported by two hybrid receptors could be inhibited by both RANTES and monocyte chemoattractant protein-1, chemokines specific to CCR5 and CCR2B, respectively; and second, the chemokine specificity for inhibition of envelope-mediated cell fusion matched the agonist profile of these hybrid receptors. These data shed new light on the structural determinants involved in these distinct activities of CCR5 and may have important implications for the development of CCR5-targeted anti-viral compounds.

CC chemokine receptor 5 (CCR5),1 a seven-transmembrane domain (TMD7) G-protein coupled receptor for the CC chemokines macrophage inflammatory protein (MIP)-1α, MIP-1β, and RANTES (regulated upon activation, normal T cell expressed and secreted) (1–3), plays a critical role in transmission and pathogenesis of human immunodeficiency virus (HIV)-type 1 infection. When co-expressed with CD4, CCR5 serves as a co-receptor for entry of macrophage (M)-tropic and dual-tropic strains of HIV-1 (4–8). The importance of CCR5 in HIV-1 transmission is highlighted by the findings that individuals homozygous for a 32-base pair deletion in CCR5 have greatly reduced susceptibility to HIV-1 infection; the protein encoded by the defective CCR5 gene cannot be detected on the cell surface and is nonfunctional as a fusion co-receptor (9–13). Of note, these individuals do not have any detectable immunological defect, suggesting that a strategy designed to mimic a CCR5 null mutation may prove to be a viable therapeutic approach.

The observation that MIP-1α, MIP-1β, and RANTES activate CCR5 (1–3) and also suppress infection and fusion by M-tropic HIV-1 strains (9–14) suggests that the suppressive effects of chemokines are exerted (at least in part) by blocking sites on CCR5 involved in interaction with the HIV-1 envelope (Env) glycoprotein (gp). In support of this, soluble gp120 complexed to CD4 has been shown to inhibit binding of radiolabeled chemokine to CCR5-expressing cells (15, 16). Furthermore, it is possible some of the HIV-inhibitory effects of the CC chemokines may be due to down-modulation of CCR5 from the cell surface. Thus, identification of CCR5-HIV-1 interaction sites and knowledge of whether they overlap with those required for chemokine interaction are critical for understanding HIV-1 transmission and pathogenesis and may help guide the design of novel anti-HIV-1 compounds that target this interaction.

To examine the molecular determinants of human CCR5 that are important in supporting HIV-1 entry, recent studies (17–19) have examined the co-receptor activity of chimeric mol-

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1 The abbreviations used are: CCR, CC chemokine receptor; HIV, human immunodeficiency virus; MIP, macrophage inflammatory protein; M-tropic, macrophage tropic; Env, envelope; gp, glycoprotein; TMD, transmembrane domain; MCP, monocyte chemoattractant protein; PCR, polymerase chain reaction; HEK, human embryonic kidney; PBS, phosphate-buffered saline.

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EXPERIMENTAL PROCEDURES

Creation of Constructs Expressing Wild Type and Chimeric Human CCR5 and CCR2B Receptors—The coding regions of CCR5 and CCR2B were amplified by polymerase chain reaction (PCR) from genomic DNA, subcloned into the Smal restriction site of a pBluescript SKII+ vector (Stratagene) that had been modified by deleting the sequences in the multiple cloning site that span from the PstI to AccI restriction sites of pBluescript SKII+. The conserved ClaI and EcoRI (see Fig. 1) restriction sites of CCR5 and CCR2B were used to construct the chimeras (see Fig. 2). The chimeras are named according to the origin of their four extracellular segments. For example, chimera 5552 is an example where the fourth extracellular domain (e3) loop of CCR5 has been exchanged with that from CCR2B. For HIV-1 Env-mediated cell fusion assays using the vaccinia-based system, the receptor constructs were transferred into the NotI and Xhol sites of pBlueScript SKII+ so that the translation initiation sites are adjacent to the T7 promoter; expression was achieved by vaccinia-encoded bacteriophage T7 RNA polymerase (see below). Point mutants were introduced into the e3 loop of chimera 5552 by 1) digesting chimera 5552 in pBluescript SKII+ with restriction endonucleases NotI and HincII; 2) using PCR with the T3 primer in conjunction with a primer that included an EcoRI linker and the sequence encoding the desired mutation(s). The PCR template was the 5552 pBluescript SKII+ plasmid that had been digested with NotI and HincII; and 3) digesting the amplified PCR product with the restriction endonucleases EcoRI and Xhol and then ligating it into a CCR5-pBluescript SKII+ plasmid that had also been digested with EcoRI and Xhol. The fidelity of all the receptor constructs was verified by DNA sequencing.

Creation of Cell Lines Stably Expressing Wild Type and Chimeric Human CCR5 and CCR2B Receptors—Receptor DNA was transferred into the NotI and Xhol sites of the hygromycin-selectable, stable episomal vector pCEP4 (Invitrogen). Human embryonic kidney (HEK) 293 cells (107) grown to log phase in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum were electroporated with 20 µg of plasmid DNA. Multiple hygromycin-resistant colonies were picked and expanded in 150 µl/ml of hygromycin (Calbiochem).

Intracellular [Ca2+] Measurements—HEK 293 transfectants (107/ml) were suspended in Hanks’ buffered saline solution with Ca2+ and Mg2+ and 10 mM Hepes, pH 7.4, containing 2.5 µM Fura-2 for 30 min at 37 °C in the dark. The cells were subsequently washed twice in phosphate-buffered saline (PBS) and then resuspended in Hanks’ buffered saline solution with Ca2+ and Mg2+ at 2 × 106 cells/ml. 2 ml of the cell suspension were placed in a continuously stirred cuvette maintained in a fluorimeter F4500 (Hitachi). [Ca2+] changes elicited at varying concentrations of human CC chemokines (R&D) from transfected HEK 293 cells was determined. Fluorescence was monitored at λex = 340 nm, λem = 380 nm, and λem = 340 nm, and the data are presented as the relative ratio of fluorescence at 340 and 380 nm.

Cells and Viruses—HeLa, BSC-1, and NIH 3T3 cell lines (ATCC) were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Recombinant vaccinia viruses were grown in BSC-1...
Flow cytometry analysis using a polyclonal rabbit antiserum against the NH₂ terminus of CCR5 yielded the following mean fluorescence m 96-well microtiter plate in the presence of 40 Fura-2-loaded HEK 293 cells stably expressing wild type or chimeric receptor were recorded as being either strongly positive (cells, and purified stocks were prepared by standard procedures (20).

moter were transfected into NIH 3T3 cells using DOTAP lipofection mediated cell fusion (21). To prepare target cells, plasmid DNA (in determined by a vaccinia-based reporter gene assay quantitating Env-

FIG. 2. Wild type CCR5 and CCR2B and chimeric CCR2B/CCR5 chimeras. The designation of each receptor tested and a schematic illustration of its sequence composition (white, CCR5 sequence; black, CCR2B sequence) are shown on the top of the Fig. A, fusion activity was assessed as described under “Experimental Procedures.” The results for each co-receptor are expressed as the percentage of β-galactosidase activity relative to the value obtained with CCR5 (defined as 100% for each Env). The values (± standard deviation) were derived from at least three independent experiments. In each independent experiment, samples were analyzed in duplicate. The β-galactosidase activities (optical density × 1000/min) obtained with CCR5 for each Env were as follows: Ba-L, 120 ± 3; JR-FL, 260 ± 30; ADA, 385 ± 5; SF162, 350 ± 2.5; and 89.6, 80 ± 10. Flow cytometry analysis using a polyclonal rabbit antiserum against the NH₂ terminus of CCR5 yielded the following mean fluorescence intensities for cells expressing the indicated receptors: none, 13; CCR2B, 9; CCR5, 95; 5552, 50; 5525, 83; and 5522, 83. The ability of agonists (final concentration, 50 nM) specific for CCR5 (MIP-1α, MIP-1β, and RANTES) or CCR2B (MCP-1) to mobilize intracellular calcium from Fura-2-loaded HEK 293 cells stably expressing wild type or chimeric receptor were recorded as being either strongly positive (+ +), weakly positive (+), or negative (−). The results are derived from a minimum of six different experiments, with at least five different HEK 293 clones expressing the receptor construct. Detailed calcium transients are shown in Fig. 4.

cells, and purified stocks were prepared by standard procedures (20).

Assays of HIV Fusion Co-receptor Activity—Co-receptor activity was determined by a vaccinia-based reporter gene assay quantitating Env-mediated cell fusion (21). To prepare target cells, plasmid DNA (in pBluecript KSII+) containing the insert sequences encoding wild type CCR5, CCR2B, or chimeric/mutant constructs linked to the T7 promoter were transfected into NIH 3T3 cells using DOTAP lipofection reagent (Boehringer Mannheim). After 4 h of incubation at 37 °C, the transfected cells were coincubated with the T7F7–3 encoding bacteriophage T7 RNA polymerase under the control of a natural vaccinia virus early/late promoter (22) and vCB-3 (23) encoding human CD4 under control of a synthetic strong early/strong late vaccinia promoter; the multiplicity of infection was 10 plaque-forming unit/cell for each virus. The control in each of these experiments represented NIH 3T3 cells transfected with the vector lacking inserts and infected with a vaccinia recombinant vaccinia viruses. For preparation of effector cells expressing HIV-1 Env, HeLa cells were co-infected with the recombinant vaccinia virus vCB-21R encoding β-galactosidase under control of the T7 promoter (24) and one of the following vaccinia recombinants containing the indicated Env linked to the synthetic strong early/strong late promoter (25): vCB-43, Ba-L (M-tropic); vCB-45, Ba-L (M-tropic); vCB32, SF-162 (M-tropic); vCB-28, JR-FL (M-tropic); and vCB-16, IIB uncleaved (Unc, rendered nonsusceptible by deletion of the gp120/gp41 cleavage site). For the dual-tropic 89.6 Env, the gene was cloned into plasmid pSC59 containing the synthetic strong early/strong late vaccinia promoter2 and was expressed on HeLa cells by transfection with the vaccinia-encoded proteins, then washed, and resuspended in Earle’s modified Eagle’s medium with 2.5% fetal bovine serum. Duplicate samples containing 10⁵ target cells (expressing CD4 plus the indicated chemokine receptor constructs; the cells were incubated for 30–45 min at 37 °C and then mixed with Env-expressing effector cells. To monitor the effect of chemokines on the vigorous co-receptor activity of wild type CCR5, HeLa cells were co-infected with vCB-21R and HIV-1 Env-encoding vaccinia virus in the presence of Ara-C (40 μg/ml). The drug inhibits vaccinia virus late gene expression and therefore reduces the expression of Env on HeLa cell, thereby reducing the fusion activity and facilitating measurement of inhibitory effects of chemokines.

RESULTS AND DISCUSSION

Co-receptor Activity of Wild Type CCR5, CCR2B, and CCR5/CCR2B Chimeras—In the vaccinia-based cell fusion assay, CCR5 functioned as a fusion co-receptor for Envs from several macrophage-tropic HIV-1 strains, as well as the dual-tropic strain 89.6; among the Envs tested, CCR2B functioned only for 89.6 (see Fig. 2). These results are consistent with previous reports using a variety of experimental systems (4–8).

To study the molecular determinants of CCR5 that support HIV-1 fusion co-receptor activity, we exploited the fact that most of the divergent regions between CCR5 and CCR2B are clustered in the extracellular amino acids (the NH₂-terminal segment before TMD1 and the e1, e2, and e3 loops) and the carboxyl-terminal cytoplasmic tail (Fig. 1). The contributions of these divergent regions to CCR5 co-receptor activity were assessed by creating a series of chimeras in which the following regions of CCR5 were replaced with the corresponding regions of CCR2B (Fig. 1): 1) the NH₂ terminus to the end of TMD3, including the e1 loop (chimera 2255); 2) the second intracellular loop to the fifth amino acid in the e3 loop, including the e2

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loop (chimera 5525); 3) the sixth amino acid in the e3 loop to the end of the carboxyl-terminal cytoplasmic tail (chimera 5552). Because the first five amino acids of the e3 loop of CCR5 and CCR2B are identical, chimera 5552 is suited to test the importance of the e3 loop of CCR5 in supporting HIV-1 entry. We also created a complementary set of chimeras in which these regions of CCR2B were replaced with the corresponding regions of CCR5 (5522, 2252, and 2225). These chimeras are identical to a set of chimeras previously reported by Rucker et al. (17), who also used the conserved EcoRI and ClaI restriction endonuclease sites to switch homologous regions between CCR5 and CCR2B.

Fig. 2 shows that these CCR5/CCR2B chimeras exhibited a 75–100% decrease in co-receptor activity for Envs from the M-tropic strains. The fusion co-receptor defects of the chimeric molecules were not due to loss of cell surface expression, as judged by one direct (flow cytometry) and two indirect criteria. First, all chimeras except 5522 exhibited some co-receptor activity for the 89.6 Env (Fig. 2). Second, flow cytometry analysis using an antiserum directed against the NH2-terminal extracellular region demonstrated that all chimeras containing this region were expressed at levels comparable with wild type CCR5 (Fig. 2 legend). Finally, the three receptor constructs that include the NH2 terminus of CCR2B (2255, 2225, and 2225) all mobilized calcium in response to one or more CC chemokines (summarized in Fig. 2 and illustrated in detail in Fig. 4). Our results therefore suggest that the fusion co-receptor activity of CCR5 involves several regions, including the NH2 terminus and/or the e1 loop, the e2 loop, and the e3 loop.

Our results show many parallels with previous studies of CCR5/CCR2B chimeras, in which the structural determinants on CCR5 involved in fusion co-receptor activity were found to be complex and to involve multiple extracellular regions (17, 18). There is good agreement on the loss of M-tropic co-receptor activity with chimeras 5522, 2255, 2252, and 2225. However, there are some significant differences between our findings and the previous studies. In particular, we observed considerable loss of M-tropic fusion co-receptor activity for chimeras 5525 and 5552, suggesting importance for the e2 loop and, to a greater extent, the e3 loop; these results are in contrast to the report of minimal effects of such changes on fusion co-receptor activity with a M-tropic Env (17). Atchinson et al. (18) have also analyzed human-murine CCR5 chimeras; however, because murine and human CCR5 have identical sequences in the e3 loop, chimeras between murine/human CCR5 cannot reveal the importance for this region in viral entry. Discrepancies have also been reported regarding the CCR5 extracellular NH2-terminal region; although the importance of this domain has been clearly demonstrated by analysis of chimeras and site-directed mutants (17–19), varying results have been reported for the activities of certain chimeras with similar structures. Presumably differences in the alternative expression and assay systems account for these discrepancies. For example, the target cells used by Rucker et al. (17) were quail QT6, whereas we
used NIH3T3 cells; the gene reporter assay systems are also different (luciferase versus β-galactosidase). Atchison et al. (18) quantitated HIV-1 entry into COS cells transiently co-transfected with CD4 and chemokine receptors by measuring intracellular expression of the viral capsid protein p24 by fluorescence-activated cell sorting. The assay (chloramphenicol acetyltransferase activity) and cell expression system (HeLa-CD4 and CF2Th canine thymocytes) used by Farzan et al. (19) is also different from those used in this study.

In interpreting our results for diminished M-tropic fusion co-receptor activity for chimera 5552, it must be noted that this molecule contains not only the e3 loop of CCR2B but also TMD7 and the carboxyl-terminal cytoplasmic tail. The residues in TMD7 of CCR5 and CCR2B are identical; however, in the the 51 residues of the carboxyl-terminal cytoplasmic tail of CCR5, 20 differences exist between the two receptors, of which only three are conservative (Fig. 1). The possibility must therefore be considered that these differences contribute to the reduction of M-tropic co-receptor activity in chimera 5552.

It is striking that a chimera containing the NH2 terminus of CCR2B and the extracellular loops of CCR5 (chimera 5222) can fully support HIV-1 entry (17–19), whereas chimera 5552 (this study and Ref. 17) does not. Conceivably, despite the high degree of sequence homology between CCR5 and CCR2B, “artificial” receptors created by exchanging different regions of CCR5 and CCR2B may have unusual folding patterns that could account for the unpredictable co-receptor activities that we and others have observed.

Importance of the CCR5 e3 Loop for M-tropic Fusion Co-receptor Activity—In comparing the extracellular domains of CCR5 and CCR2B, the e3 loop is one of the most conserved. In this region only six of the 23 residues of CCR5 are different from CCR2B, and of these one is a conserved substitution (Fig. 1). To examine the importance of individual residues in the e3 loop of CCR5 for M-tropic co-receptor activity, we created site-directed mutants of chimera 5552 in which the CCR2B-specific amino acids in this region were replaced with those present at the corresponding positions in CCR5 (Fig. 1). For example, in chimera 5552Δ32, we changed the CCR2B-specific residues Glu and Gln to the CCR5-specific residues Ser and Arg, respectively. Flow cytometry analysis verified that each of the mutants was expressed at the cell surface at levels comparable with wild type CCR5 (see Fig. 3 legend). The effects of these restorative mutations on fusion co-factor activity are shown in Fig. 3 for three M-tropic Env s. Substituting the amino acid Glu → Ser (chimera 5552Δ2) had no effect on M-tropic co-receptor function, whereas substituting amino acid Gln → Arg (5552Δ5) resulted in modest restoration of activity. Although chimera 5552Δ2,5 contains the amino acid substitutions present in both chimeras 5552Δ2 and 5552Δ5, this chimera also had a minimal effect on restoring the co-receptor activity of CCR5 (Fig. 3). We also created a construct (chimera 5552Δ1–5) in which the sequence is entirely that of CCR5 from amino acids 1–278. Nearly complete restoration of M-tropic fusion co-receptor activity was observed; indeed in some experiments complete restoration of fusion co-receptor activity was observed (data not shown). Because chimera 5552Δ1–5 has the carboxyl-terminal tail of CCR2B, the differences in this intracytoplasmic region must not be significant for co-receptor function. We conclude that amino acid residues in the e3 loop of CCR5 that differ from those of CCR2B make important contributions to CCR5’s fusion co-receptor activity for M-tropic Envs. It is conceivable that additional residues in the e3 loop of CCR5 may also play an important role in supporting HIV-1 entry.

Agonist Selectivity of CCR5/CCR2B Chimeras—We next examined the structural determinants on CCR5 involved in chemokine agonist activity. Using transfectants of the HEK293 cell line, the CCR5/CCR2B chimeras were analyzed for their ability to mobilize calcium in response to chemokine ligands (Figs. 2 and 4). As previously reported, the wild type receptors CCR5 (1–3) and CCR2B (26–28) were activated in response to distinct nonoverlapping sets of agonists. Thus MIP-1α, MIP-1β and RANTES potently activated CCR5, whereas MCP-1 and MCP-3 were inactive (Figs. 2 and 4A); MCP-1 and MCP-3 were potent activators of CCR2B, whereas MIP-1α, MIP-1β, and RANTES were inactive (Figs. 2 and 4B and data not shown). Chimeras 2252 and 2255 demonstrated hybrid agonist specificities compared with the wild type receptors. Thus MIP-1α, RANTES, MCP-1, and MCP-3 were potent agonists for chimera 2252, whereas the signaling response with MIP-1β was weak (Figs. 2 and 4C). MIP-1α, MIP-1β, and RANTES were potent agonists for chimera 2255, and MCP-1 and MCP-3 showed activity at higher concentrations (Figs. 2 and 4D). Chimaera 2225 had a chemokine selectivity similar to that of wild type CCR2B; MCP-1 was active, whereas MIP-1α, MIP-1β, and RANTES were not (Figs. 2 and 4E). With chimeras 5552, 5522, and 5525 we were unable to detect intracellular calcium mobilization with any of the CC chemokines (Fig. 2). We note that hybrid agonist selectivities (MCP-1 and MIP-1β) have been reported for chimera 2255 (Ref. 18) as well as for another chimera containing the NH2 terminus of CCR2B and the extracellular loops of CCR5 (19).

Chemokine Sensitivity of M-tropic Fusion Co-receptor for the CCR5/CCR2B Chimeras—The hybrid agonist specificities observed for chimeras 2252 and 2255 suggested the importance of testing the effects of CC chemokines on fusion co-receptor activity; such analyses have not been reported in previous studies of CCR5/CCR2B chimeras. Fig. 5 shows results with the ADA Env, which displayed the greatest activity of the M-tropic Envs.
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for these chimeras. With wild type CCR5, dose-dependent inhibition of fusion was observed with RANTES but not with MCP-1, as expected (Fig. 5A). Consistent with the hybrid agonist activities described above, fusion mediated by chimeras 2252 and 2255 was inhibited in dose-dependent fashion by both RANTES and MCP-1 (Fig. 5, B and C). These results suggest that at least some common structural determinants of CCR5 are involved in both agonist activity and co-receptor function.

CCR5 ligands therefore probably exert their suppressive effects on HIV-1 fusion and entry by interfering with Env-CCR5 interactions. This notion is supported by the direct demonstration of gp120 blocking of radiolabeled chemokines to CCR5-expressing cells (15, 16). It is also possible that CC chemokines trigger down-regulation of CCR5 (as has been shown for other chemokine/receptor interactions (29)), rendering it unavailable for use as a fusion co-receptor. It should be emphasized that our studies do not resolve the questions of whether HIV-1-CCR5 interactions result in receptor activation and whether such activation is essential for viral fusion. Recent studies argue that at least some common structural determinants of CCR5 totally convergent, a narrower spectrum of antagonists that specifically block CCR5-Env interactions can be envisioned. Additional studies will be required to develop a more detailed model of the HIV-1 Env and CC chemokine interaction sites on CCR5, including studies with the recently described CCR5 antagonists (30).

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