JOURNAL OF VIROLOGY, Sept. 1997, p. 6305–6314 Vol. 71, No. 9
0022-538X/97/$04.00+0

Two Distinct CCR5 Domains Can Mediate Coreceptor Usage by Human Immunodeficiency Virus Type 1

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Received 11 March 1997/Accepted 29 May 1997

The chemokine receptor CCR5 is the major fusion coreceptor for macrophage-tropic strains of human immunodeficiency virus type 1 (HIV-1). To define the structures of CCR5 that can support envelope (Env)-mediated membrane fusion, we analyzed the activity of homologs, chimeras, and mutants of human CCR5 in a sensitive gene reporter cell-cell fusion assay. Simian, but not murine, homologs of CCR5 were fully active as HIV-1 fusion coreceptors. Chimeras between CCR5 and divergent chemokine receptors demonstrated the existence of two distinct regions of CCR5 that could be utilized for Env-mediated fusion, the amino-terminal domain and the extracellular loops. Dual-tropic Env proteins were particularly sensitive to alterations in the CCR5 amino-terminal domain, suggesting that this domain may play a pivotal role in the evolution of coreceptor usage in vivo. We identified individual residues in both functional regions, Asp-11, Lys-197, and Asp-276, that contribute to coreceptor function. Deletion of a highly conserved cytoplasmic motif rendered CCR5 incapable of signaling but did not abrogate its ability to function as a coreceptor, implying the independence of fusion and G-protein-mediated chemokine receptor signaling. Finally, we developed a novel monoclonal antibody to CCR5 to assist in future studies of CCR5 expression.

The initial events in the infection of target cells by human immunodeficiency virus type 1 (HIV-1) include receptor binding and membrane fusion. The viral envelope glycoprotein (Env) mediates both processes in conjunction with at least two different cellular molecules. While CD4 on the cell surface binds to Env with high affinity, CD4 alone is not sufficient to trigger the conformational changes in Env that lead to fusion of viral and cellular membranes (52, 53). Members of the chemokine receptor family of G-protein-coupled receptors (GPCRs) are required for this final fusion event to occur, but the mechanism by which these receptors allow Env to mediate mixing of the lipid bilayers has not yet been elucidated.

The chemokine receptor family members that are currently implicated as coreceptors in HIV-1 entry include CCR5, CCR3, CCR2b, and CXCR4 (3, 8, 14, 22, 24, 25, 28). While CD4 binds all HIV-1 Env proteins, the fusogenic activity of each chemokine receptor is limited to a subset of Env proteins, thereby helping to explain the molecular basis for the tropism of different HIV-1 strains. Thus, CXCR4 serves as the primary fusion coreceptor for T-cell-tropic (T-tropic) strains (28), while CCR5 has been identified as the primary fusion coreceptor for macrophage-tropic (M-tropic) strains of HIV-1 (3, 14, 22, 24, 25). M-tropic strains of HIV-1 are preferentially transmitted from one infected individual to another via sexual contact, vertical transmission, and direct blood transmission (17, 56, 57, 60). Individuals who lack functional CCR5 genes are highly resistant to HIV-1 transmission, demonstrating the importance of CCR5 for HIV-1 infection in vivo (19, 36, 41, 51).

Since CCR5 appears to be critical for HIV-1 infection, understanding the molecular mechanism by which M-tropic strains utilize CCR5 is highly relevant for understanding HIV-1 transmission and pathogenesis. The way in which Env interacts with the chemokine receptors remains to be defined, but a complex structure on CCR5 has previously been implicated in this interaction (5, 49). A trimeric interaction between CD4, Env, and the chemokine receptors has been detected (40, 55, 59), but the consequences of this interaction are not clear. One possibility is that a physical association with CCR5 triggers the induction of Env into its fusogenic conformation. Alternatively, Env-CCR5 interactions might result in signal transduction and internalization of the receptor into endosomes. Either activity has the potential to impact the subcellular environment and location in which viral fusion occurs.

In this study, we examined the structural determinants of CCR5 coreceptor function by using a sensitive gene reporter cell-cell fusion assay. We found that CCR5 homologs derived from several primate species functioned as fusion coreceptors, whereas murine CCR5 did not. Analyses of receptor chimeras demonstrated that while the first 20 amino acids of CCR5 were sufficient to confer coreceptor activity to divergent, nonfusogenic chemokine receptors, domains in the extracellular loops form a distinct structure that also contributes to coreceptor function. Site-directed mutagenesis identified residues in the amino-terminal domain (Asp-11), the second extracellular loop (Lys-197), and the third extracellular loop (Asp-276) of CCR5 that are critical for coreceptor function for the envelopes of some virus strains. The sensitivity of dual-tropic Envs to changes in the amino-terminal domain suggests that this region may be critical for the evolution of coreceptor usage in vivo. Furthermore, we show that the function of CCR5 as an HIV-1 fusion coreceptor does not require its G-protein-medi-

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ated signaling capability. Finally, we developed a highly specific monoclonal antibody, 12D1, to the amino-terminal domain of CCR5 to assist in future studies of CCR5 expression.

MATERIALS AND METHODS

CCR5 chimeras and mutants. To create chimeras, complementary regions of the two parental receptors designed to compose the chimera were amplified from cDNA templates in PCR reactions catalyzed by *Pfu* polymerase. Since the resulting DNA fragments contained blunt ends, they were phosphorylated and ligated together. The ligation product encoding the desired chimeric receptor was then amplified by using the appropriate upstream and downstream primers from the respective parental receptors and cloned into the TA vector. All PCR-amplified regions were sequenced. The final cDNA encoding the chimeric receptor was subcloned into the pcDNA3 expression vector, using unique sites introduced into the PCR primers. Point mutants were prepared by using a similar approach in which a codon for alanine was substituted for that encoding the targeted charged residue in a PCR primer. Following PCR-ligation-PCR, candidates were screened for the presence of the mutant codon by nucleotide sequence analysis. Multiple point mutations were created by swapping restriction fragments from the various single CCR5 mutants. Deletion mutants were prepared by using primers to amplify the segments upstream and downstream of the mutation and performing PCR-ligation-PCR. CCR5 homologs from nonhuman primate species were isolated from genomic DNA by amplification with PCR primers designed from sequences flanking the open reading frame encoding the human receptor. Primate samples were kindly provided by Bob Andrews (Fred Hutchinson Cancer Center). The homolog from the 129 strain of mouse was amplified by using primers designed from the published mouse sequence. Additional details on cloning procedures and primer sequences can be obtained from the authors.

pREP8-Lestr plasmid has been described previously (8). Plasmid pcDNA3-Lestr was constructed by cloning the CXCR4 (Lestr) gene from plasmid pREP8-Lestr into the pDNA3 vector, using *Eco*RI and *Xho*I restriction sites. pCR3.1-JR-FL was constructed by cloning the JR-FL *env* gene from pCB28 (10) into the pCR3.1 vector, using *Eco*RI and *Xba*I restriction sites. The T7-luciferase plasmid was obtained from Promega. Plasmid pT4, providing expression of human CD4 under the control of the cytomegalovirus promoter, was provided by Dennis Kolson (University of Pennsylvania). The thrombin receptor plasmid was a gift from Peter O'Brien (University of Pennsylvania).

Viruses. The following recombinant vaccinia viruses encoding the envelope glycoproteins from a variety of HIV-1 strains (indicated in parentheses) were used: vSC60 (IIIB, BH8 clone), vCB28 (JR-FL), vCB53 (CM243), vBD3 (89.6), vCB39 (ADA), vCB43 (Ba-L), vCB32 (SF162), and vCB36 (RF) (10, 24). We also used the recombinant virus vTF1.1, encoding T7 RNA polymerase (2).

Cells. The human cervical carcinoma cell line HeLa and the human astroglioma cell line U87-MG (ATCC HTB-14) were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases. The Japanese quail fibrosarcoma cell line QT6-C5 (QT6; ATCC CRL-1708) and the human kidney cell line 293T were provided by Paul Bates, University of Pennsylvania. A QT6 cell line expressing human CD4, QT6-T4, was created by transfecting QT6 cells with pT4 and maintaining cells under continuous selection in medium containing G418 (600 μg/ml). All cells were maintained in Dulbecco's modified Eagle medium (high glucose) supplemented with 10% fetal bovine serum, 2 mM glutamine, and 2 mM penicillin-streptomycin.

Gene reporter fusion assay. To quantitate cell-cell fusion events, we used a version of the gene reporter fusion assay described by Nussbaum et al. (46) and modified by our laboratory (24). T7 RNA polymerase and Env proteins were introduced into effector HeLa cells by infection with recombinant vaccinia viruses at a multiplicity of infection of 10 for 2 h. Effector cells were then trypsinized, washed with phosphate-buffered saline (PBS), resuspended in medium, and incubated at 32°C overnight in the presence of rifampin (100 μg/ml). Target cells were transfected with pT4, T7-luciferase, and coreceptor constructs as indicated in the text and figure legends. Target cells were transfected in 24-well plates by transfection of 1 to 2 μg of each plasmid, using the calcium phosphate precipitation method. The transfectant was removed after 4 to 6 h, the medium was replaced, and the cells were incubated at 37°C overnight. To initiate fusion, target cells were resuspended in medium with rifampin and cytosine arabinofuranoside, and 10^5 effector cells were added to each well. Fusion was allowed to occur at 37°C for 8 h before lysis in 150 μl of 0.5% Nonidet P-40 (NP-40) in PBS and assay for luciferase activity by using commercially available reagents (Promega). Luciferase activity was quantitated with a Wallac 1450 Microbeta luminometer detector within the linear range of luciferase activity detection. Absolute values of results are indicated in the figure legends and should be evaluated based on the background levels of fusion without coreceptors present (i.e., signal-to-noise ratio). Conditions for the cell-cell fusion assay were designed to maximize sensitivity in order to detect all regions of CCR5 that may contribute to coreceptor function.

Ca^{2+} mobilization assays. Response to ligand was determined in transiently transfected human 293T cells. Cells were transfected with the desired coreceptor(s) for 6 h, medium was replaced, and cells were allowed to express overnight. Medium was replaced with Dulbecco's PBS containing calcium and magnesium (BioWhittaker) plus 2.5 μM Fura-2/AM (Molecular Probes), and cells were incubated at 37°C in the dark for 1 h. Cells were allowed to efflux for 15 min in phenol red-free RPMI 1640 (Gibco-BRL) before cells were removed from the plate by manual disruption. Cells were centrifuged and resuspended in phenol red-free RPMI 1640 at 2×10^6 cells/ml and were warmed at 37°C for 15 min before measurement of ligand response. Ca^{2+} mobilization was measured in an Aminco-Bowman luminescence spectrometer in a constantly stirred cuvette and in a volume of 1.5 ml. Cells were excited at 340 nm, emission was read at 510 nm, and Ca^{2+} concentration was calculated as previously described, using an assumed dissociation constant of 224 (34). MIP1α was used at concentrations of up to 666 ng/ml (84 nM) as a ligand for CCR5 and had no background activity on 293T cells in this assay. The BB-10010 variant of human MIP1α with improved solubility (37) was obtained from British Biotech. Thrombin-activating peptide 4 was kindly provided by Mike Orsini (University of Pennsylvania) and was used at a final concentration of 27 μM.

Infection studies. Viral stocks were prepared as previously described by transfecting 293T cells with plasmids encoding JR-FL *env* (pCR3.1-JR-FL) and the NL4-3 luciferase virus backbone (pNL-Luc-E^-R^-) (16). The resulting supernatant was sterile filtered and stored at −80°C. For infection, U87-MG cells were prepared in 24-well plates and transfected with pT4 and the desired coreceptor. Medium was changed after 6 h, and cells were allowed to express overnight. Cells were infected the next day with 500 μl of viral supernatant. Medium was changed the following day, and 0.5 ml of additional medium was added 1 day prior to harvest of cells. Cells were lysed at 4 days postinfection by resuspension in 150 μl of 0.5% NP-40–PBS, and 20 μl of the resulting lysate was assayed for luciferase activity.

Immunoprecipitation. Lysates containing an N-terminal hemagglutinin (HA)-tagged form of CCR5 in pcDNA3 (49) were prepared by infecting a T25 flask of QT6 cells with vTF1.1, encoding T7 polymerase, to enhance expression, transfecting cells 2 h later with 15 μg of CCR5-HA plasmid by calcium phosphate precipitation, and allowing expression overnight. Cells were lysed in 1 ml of lysis buffer (0.1 M Tris 8.0, 0.1 M NaCl, 1 mM $CaCl_2$, 1% Triton X-100), cell debris was removed by centrifugation, and the supernatants were stored at −80°C until use. Immunoprecipitations were performed by using 22 μl of cell lysate, 40 μl of protein A beads (immunoglobulin G binding capacity, 4 mg/ml), and 450 μl of immunoprecipitation binding buffer (10 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% NP-40). One microliter of 12D1 (anti-CCR5) ascites fluid was used with 3 μl of goat anti-mouse secondary antibody (Sigma), and 5 μl of 12CA5 (anti-HA tag) ascites fluid was used without secondary antibody. Protein was detected as previously described (8, 49) by incubation overnight at 4°C, washing three times with PBS, boiling in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis loading dye containing urea, and electrophoresis through SDS–10% polyacrylamide-urea gels. Samples were transferred to polyvinylidene difluoride membranes, probed with 12CA5 ascites fluid (1:1,000), and illuminated by using the Amersham ECL reagent.

Nucleotide sequence accession numbers. Primate and murine CCR5 sequences have been submitted to GenBank under accession no. AF005658 to AF005663.

RESULTS

Primate, but not murine, homologs of CCR5 function as fusion coreceptors. The ability of CCR5 to function as a fusion coreceptor appears to be cell type independent, since several nonprimate cell lines that are normally resistant to HIV-1 entry and fusion (4, 11, 43), including the QT6 quail fibrosarcoma cell line used in this study, are rendered susceptible to HIV-1 Env-mediated membrane fusion and virus entry by coexpression of CCR5 and CD4 (3, 14, 22, 24, 25). To study the mechanism by which CCR5 functions as an HIV-1 fusion coreceptor, we used a gene reporter cell-cell fusion assay to assess the ability of CCR5 variants to support Env-mediated fusion (24, 46). In this assay, CD4 and the coreceptor of interest are expressed in target QT6 cells by transient expression with a T7-luciferase reporter construct. After 24 h, the target cells are mixed with HeLa effector cells that express the envelope protein of interest and T7 polymerase from vaccinia virus-based vectors. Fusion of the two cell populations results in cytoplasmic mixing and expression of the luciferase reporter. Previous use of this system has demonstrated concordance between cell-cell fusion and the ability of HIV or simian immunodeficiency virus (SIV) to enter a cell (24, 26, 42, 49, 51). The conditions used in these experiments were designed to maximize the sensitivity of detecting cell-cell fusion events in order to identify receptors or regions of receptors that con-

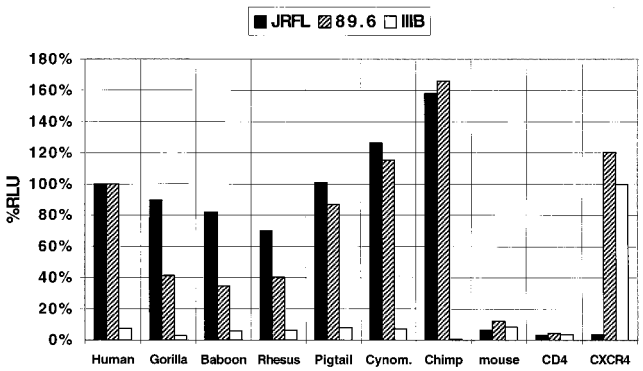

FIG. 1. Coreceptor function of CCR5 derived from human, nonhuman primate, and mouse cells. QT6 cells transfected with CD4, the indicated coreceptor, and luciferase under control of the T7 promoter were mixed with HeLa effector cells infected with vaccinia virus vectors expressing either T-tropic (BH8), M-tropic (JR-FL), or dual-tropic (89.6) Env proteins. Effector cells were also infected (multiplicity of infection of 10) with vTF1.1, which expresses T7 polymerase under the control of the vaccinia virus late promoter. Cells were allowed to fuse for 8 h before lysis in 0.5% NP-40–PBS and assay for luciferase activity. Results are expressed as relative light units (RLU) detected by a Wallac 1450 Microbeta luminometer detector within the linear range of luciferase activity detection and represent a single experiment repeated several (>3) times with similar results. All wells received CCR5 or CCR5 homologs in pcDNA3 as indicated except for CXCR4, which received plasmid pREP8-Fusin, encoding human CXCR4, and the negative control CD4, which received the pcDNA3 vector in place of CCR5. All wells received plasmid pT4. Results for JR-FL and 89.6 are normalized to the reporter activity measured with wild-type CCR5 (100% represents 46,330 and 41,424 RLU, respectively), and results for IIIB are normalized to the reporter activity measured with CXCR4 (100% represents 48,131 RLU). Cynom., cynomolgus.

tribute to Env-mediated membrane fusion. Due to the diverse nature of the receptors studied, no single immunological reagent could be used to accurately quantitate surface expression. Therefore, levels of fusion with any particular construct should be compared to the level of fusion obtained in the absence of chemokine receptor. Differences in fusion efficiency observed between chemokine receptor mutants will require a standardized method of detecting cell surface levels in order to be fully understood.

Regions of the CCR5 molecule that differ between functional and nonfunctional animal homologs offer a starting point for identifying regions of the molecule that functionally interact with Env. Identification of homologs of CCR5 that do and do not support HIV entry will also affect animal models of HIV infection. Homologs of human CCR5 were cloned by amplifying genomic DNA templates derived from rhesus and pigtail macaques, cynomologous monkey, chimpanzee, gorilla, baboon, and mouse. Each CCR5 homolog was expressed in QT6 cells by cotransfection with plasmids encoding human CD4 and T7-luciferase. Fusion was assessed by using HeLa effector cells expressing Env proteins from the HIV-1 strains JR-FL (M tropic), 89.6 (dual tropic), and BH8 (T tropic).

Figure 1 demonstrates that, consistent with previous results, human CCR5 functioned as a fusion coreceptor for M-tropic (JR-FL) and dual-tropic (89.6), but not T-tropic (BH8), strains of HIV-1. All six nonhuman primate CCR5 homologs functioned similarly, supporting fusion with JR-FL and 89.6 but not with BH8. In contrast, the murine CCR5 homolog did not function as a fusion coreceptor for any Env tested, in agreement with previous results demonstrating that murine CCR5 lacks coreceptor activity for the M-tropic HIV-1 strain Ba-L (5). The simian CCR5 sequences were, as expected, nearly identical (>97%) to the human CCR5 sequence. In contrast, murine CCR5 is only 82% identical to human CCR5 and contains a number of amino acid changes that may contribute to its failure to function as an HIV-1 coreceptor (see Fig. 7 for comparison). Since the third extracellular loop of murine CCR5 is nearly identical to that of human CCR5, amino acid differences in the amino-terminal domain and the first and second extracellular loops are likely to account for the inability of murine CCR5 to function as an HIV-1 coreceptor.

The CCR5 amino-terminal domain supports Env-mediated fusion in the context of divergent chemokine receptors. Recent studies have shown that as few as 20 amino acids from the amino-terminal domain of CCR5 are sufficient to confer coreceptor activity to closely related receptors, including CCR2b (76% identity to CCR5) and murine CCR5 (82% identity) (5, 49). To determine the dependence of the CCR5 amino-terminal domain on other structures in the molecule, we introduced the first 20 amino acids of CCR5 into the more divergent chemokine receptors CCR1 (56% homology), CXCR2 (also known as IL-8-RB; 34% homology), and CXCR4 (33% homology) and examined the ability of each of these chimeras to support cell-cell fusion by the JR-FL, 89.6, and RF Env proteins. We have named the chimeras according to the parental molecule from which each extracellular domain is derived, designating the CC chemokine receptors by number and the CXC chemokine receptors by letter. Thus, chimera 5BBB represents the amino-terminal domain of CCR5 attached to the three extracellular loops of CXCR2 (IL8-RB), while chimera L555 is composed of the CXCR4 (Lestr/fusin) amino-terminal domain in a CCR5 background. To minimize structural changes that might prevent the correct expression of these chimeras, segments were joined at the highly conserved Cys residue present in the amino-terminal domain of all chemokine receptors (residues 20 in CCR5, 32 in CCR2b, 24 in CCR1, 39 in CXCR2, and 28 in CXCR4). As demonstrated previously, chimera 5222 supports cell-cell fusion by both JR-FL and 89.6 Env proteins (Fig. 2) (49). When the CCR5 amino-terminal domain was placed in the divergent backgrounds of CCR1 (chimera 5111), CXCR2 (5BBB), and CXCR4 (5LLL), it was still sufficient to support cell-cell fusion by JR-FL and 89.6, indicating that the first 20 amino acids of CCR5 are an important determinant of coreceptor function. In

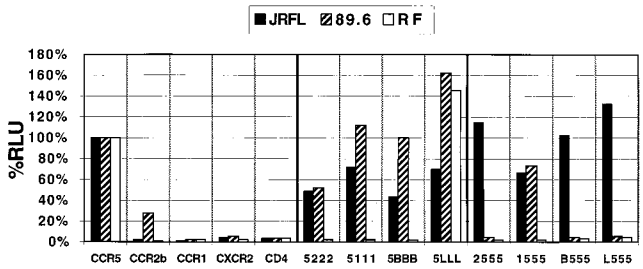

FIG. 2. Coreceptor function of chimeric chemokine receptors. Coreceptor activity was assessed by cell-cell fusion as for Fig. 1 except that QT6-T4 cells that stably express CD4 were used. One microgram of coreceptor and 2 μg of T7-luciferase were transfected into each well of cells. Receptor chimera 5111 represents CCR1 in which the amino-terminal domain up to the first conserved Cys residue is replaced with the corresponding region from CCR5. The other chimeras are designated in a similar fashion, with B representing domains from CXCR2 (IL8-RB) and L representing domains from CXCR4 (Lestr). The negative control CD4 received the pcDNA3 vector in place of a plasmid encoding a chemokine receptor. Effector cells were prepared with vaccinia viruses encoding the Env from M-tropic JR-FL (vCB28) or dual-tropic 89.6 (vBD3) or RF (vCB36) virus. Results are normalized to the reporter activity measured with wild-type CCR5 (100% represents 12,933, 9,186, and 9,833 relative luciferase units [RLU] for JR-FL, 89.6, and RF, with background [CD4] counts of 380, 320, and 278). Results represent a single experiment that was repeated multiple (>4) times with similar results.

TABLE 1. Single-residue substitutions in CCR5[a]

Mutation	Cell-cell fusion		
	JR-FL	89.6	CM243
D2A	+++	+++	+++
D11A	++	+	+
E18A	++++	+++	++++
K22A	++++	++++	++++
K26A	++++	++++	++++
R31A	++++	++++	++++
D95A	+++	++++	++++
R168A	+++	++++	++++
K171A	+++	+++	+++
E172A	+++	+++	+++
K191A	+++	+++	+++
K197A	+++	+++	+++
E262A	+++	+++	+++
R274A	++++	++++	++++
D276A	++++	++++	++++

[a] Mutations of human CCR5 are designated by residue, position, and altered residue. Thus, D11A is mutation of the aspartate residue at position 11 to an alanine residue. Mutants were scored for cell-cell fusion, as described for Fig. 1 and 4, as percentage of the wild-type level: >120% (++++), 81 to 120% (+++), 61 to 80% (++), or 41 to 60% (+). No mutant yielded results consistently below 40% of the wild-type level in cell-cell fusion assays with JR-FL, 89.6, and CM243. Similar results were obtained multiple (>4) times under a variety of conditions and in a range of cell types.

contrast, the N-terminal domain of CCR5 was not sufficient to confer coreceptor activity with RF when fused to the body of a divergent chemokine receptor other than than CXCR4. These findings indicate that the amino-terminal domain of CCR5 is not sufficient to support fusion by all strains of HIV-1.

The loops of CCR5 form a second, distinct site for Env interaction. Although the amino-terminal domain of CCR5 is clearly an important determinant of coreceptor function, it can be replaced with the corresponding regions of CCR2b and murine CCR5 without loss of coreceptor activity (5, 49). To examine the function of the CCR5 loops and their dependence on amino-terminal residues, we replaced the first 20 residues of the CCR5 amino-terminal domain with the corresponding regions of CCR2, CCR1, CXCR2, and CXCR4, all of which have very little sequence homology with the CCR5 amino-terminal domain. While chimera 1555 supported cell-cell fusion by JR-FL and 89.6 Env proteins, chimeras 2555, B555, and L555 supported fusion by JR-FL only (Fig. 2). The ability of JR-FL to utilize all of these chimeras indicates that while the amino-terminal domain plays a major role in coreceptor function, the extracellular loops of CCR5 can also function independently of the CCR5 amino terminus. The ability of 89.6 to utilize chimeras that contain the CCR5 amino terminus but not most reciprocal chimeras that contain the CCR5 loops highlights the sensitivity of this dual-tropic strain to changes in the amino-terminal domain of CCR5. The sensitivity of RF further suggests that dual-tropic Envs are more constrained in their usage of CCR5 than are CCR5-restricted Envs.

Extracellular amino acids required for coreceptor function. Our experiments with chimeric chemokine receptors implicated the participation of multiple CCR5 domains in coreceptor function. To identify residues involved in coreceptor activity, each of the 15 charged amino acids in the extracellular domains of CCR5 was individually changed to Ala by site-directed mutagenesis. We tested each mutant for coreceptor function with Envs from JR-FL, 89.6, and the M-tropic, clade E primary isolate CM243 (49). Analyses of these mutants in cell-cell fusion assays demonstrated that no individual point mutant completely abolished coreceptor activity with JR-FL, 89.6, or CM243 (Table 1). However, mutation of residue Asp-11 (D11A) consistently reduced coreceptor function to approximately 50% for the primary isolates 89.6 and CM243. No other single amino acid substitution had significant effects on Env-mediated cell-cell fusion by JR-FL, 89.6, or CM243.

The participation of multiple regions of CCR5 in coreceptor activity, combined with the observation that no single charged residue in the CCR5 extracellular domains was required for coreceptor function, prompted us to create mutants of CCR5 that combined multiple Ala substitutions in different CCR5 domains within the same molecule. Many of our combination mutants included D11A, which had the strongest effect on coreceptor function of any single-residue point mutant (Table 1). We also constructed mutants of CCR5 that mimic divergent residue changes in the extracellular regions of the nonfusogenic murine homolog. Thus, CCR5.IN contains a two-amino-acid insert (Thr-Tyr) in the amino-terminal domain of CCR5 between residues 8 and 9 that is present in murine CCR5, 31/95 has Ala substituted for residues Arg-31 and Asp-95 (Gln and Val, respectively, in the mouse homolog), and 31/95.IN has all four of these amino acid changes. Additional mutations were also tested and found to have no significant effect on fusion activity (data not shown).

We found that the double mutants 11/197 and 11/276 decreased coreceptor activity with the 89.6 Env protein to approximately 20 to 40% of wild-type CCR5 levels, while the triple mutant 11/197/276 completely abolished fusion by 89.6 (Fig. 3). Interestingly, the combination mutant 197/276 had no significant effect on fusion by 89.6, suggesting a pivotal role for Asp-11 in CCR5 utilization by this virus. Neither the two-amino-acid insert (CCR5.IN) nor substitutions at residues 31 and 95, alone or in combination, affected coreceptor function, indicating that the inability of murine CCR5 to function as a coreceptor is the result of additional amino acid differences. Effects of some mutants such as D11A and 11/197/276 on

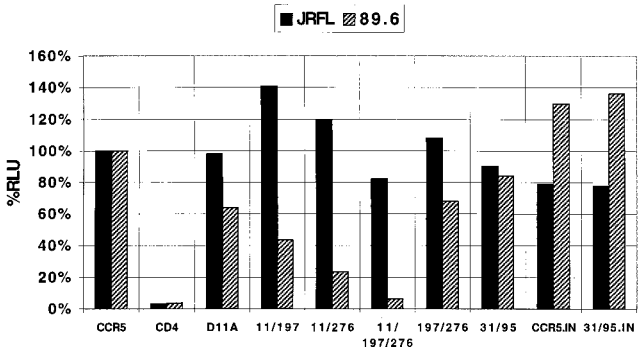

FIG. 3. Coreceptor function of CCR5 mutants containing multiple residue substitutions. Point mutations generated by alanine-scanning mutagenesis (shown in Table 1) were combined in a single CCR5 mutant as indicated. Thus, 11/197/276 represents a mutant of CCR5 containing the D11A, K197A, and D276A mutations. CCR5.IN contains a Thr-Tyr insert in the CCR5 amino-terminal domain, and 31/95.IN contains this insert along with the R31A and D95A mutations; these mutations are designed to mimic features of the nonfusogenic mouse CCR5. The negative control CD4 received the pcDNA3 vector in place of a plasmid encoding a chemokine receptor. The ability of each mutant to support membrane fusion by the JR-FL and 89.6 Env proteins was determined by using the luciferase reporter cell-cell fusion assay and QT6-T4 cells as targets as described for Fig. 1 and 2. Results are normalized to the reporter activity measured with wild-type CCR5 (100% represents 12,933 and 9,186 relative luciferase units [RLU] for JR-FL and 89.6). Results represent a single experiment that was repeated multiple (>4) times with similar results.

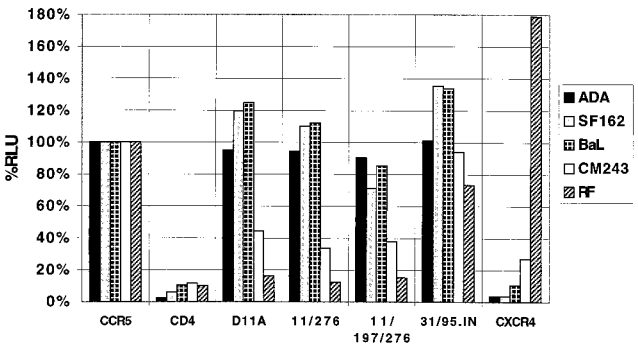

FIG. 4. Virus strain-dependent effects of mutations on coreceptor function. A subset of the mutations described in the legend to Fig. 3 was tested for the ability to support cell-cell fusion by the M-tropic Env proteins from ADA, Ba-L, SF162, and CM243 and the dual-tropic Env protein from RF. All Env proteins were expressed by using recombinant vaccinia virus vectors. CD4 and the indicated coreceptor were expressed in QT6-T4 cells. The negative control CD4 received the pcDNA3 vector in place of a plasmid encoding a chemokine receptor. CXCR4 was expressed from plasmid pcDNA3-Lestr. Results are normalized to the reporter activity measured with wild-type CCR5 (100% represents 58,219, 26,922, 11,038, 3,712, and 10,941 relative luciferase units [RLU] for ADA, SF162, BaL, CM243, and RF, respectively). Results represent a single experiment that was repeated two to four times with similar results.

JR-FL fusion activity ranged from 80 to 100% of wild-type activity.

Residues involved in CCR5 utilization by diverse viruses. Previous analysis of CCR5-CCR2b chimeras revealed differences in the way that the JR-FL and 89.6 Env proteins interact with CCR5 (49). The results in Fig. 2 and Table 1 provided additional evidence that the Envs from JR-FL, 89.6, RF, and CM243 use the CCR5 coreceptor differently. Therefore, to assess the importance of extracellular charged amino acids for a larger panel of HIV-1 Envs, we screened a number of informative double and triple CCR5 mutants against additional M-tropic (ADA, Ba-L, SF162, and CM243) and dual-tropic (RF) HIV-1 Envs in cell-cell fusion assays (Fig. 4). The M-tropic HIV-1 Envs from ADA, Ba-L, and SF162 displayed coreceptor usage patterns similar to those of JR-FL Env; none were prevented from fusing with any CCR5 mutant tested. The primary isolate CM243 was sensitive to mutation of Asp-11, but no other combination of mutations was able to eliminate its usage of CCR5. In contrast, the single amino acid mutation D11A was sufficient to completely abolish fusion by the dual-tropic Env from RF, again highlighting the sensitivity of dual-tropic Envs to changes in the amino terminus.

Receptor signaling is not required for fusion or infection. Chemokine receptors are members of the GPCR family of seven-transmembrane proteins and, as such, are capable of signal transduction after stimulation by an appropriate ligand. CCR5 transduces a signal in response to RANTES, MIP1α, and MIP1β (50), and these same chemokines have inhibitory effects on HIV-1 entry (3, 15, 22, 25). We have addressed the role of CCR5 signaling in HIV-1 fusion and viral entry by creating deletion and truncation mutants of CCR5 that are predicted to uncouple the receptor from G proteins. CCR5ΔDRY is a mutant of CCR5 that no longer contains the Asp-Arg-Tyr (DRY) motif located in the second intracellular loop of CCR5. The DRY motif is highly conserved among GPCRs, and mutation of this motif in well studied GPCRs such as rhodopsin and the α- and β-adrenergic receptors abolishes signaling activity (30–32, 58). CCR5Δtail is a mutant whose C terminus has been truncated just prior to Ser-325 so that all Ser and Thr residues in this region are removed. The C termini of many GPCRs, including the CXCR1, CXCR2, CCR2a, and CCR2b chemokine receptors, are involved in G-protein coupling, desensitization, and downregulation (7, 39, 47, 48).

Both mutants were tested for signaling activity in a Ca^{2+} mobilization assay in which transiently transfected cells that are stimulated by an appropriate ligand respond with a fluorescent emission from a Ca^{2+}-sensitive dye, Fura-2 (34). 293T cells were transfected with either CCR5, CCR5Δtail, CCR5ΔDRY, or the pcDNA3 vector. To control for transfection efficiency and cell variability, all cells were cotransfected with a plasmid expressing the thrombin receptor, a GPCR that shares no ligands with the chemokine receptors (9). As shown in Fig. 5A, CCR5Δtail continued to respond to the ligand MIP1α (70 to 100% of wild-type levels [data not shown]), while CCR5ΔDRY failed to signal. Control 293T cells transfected with the pcDNA3 vector or left untransfected failed to signal with any concentration of β-chemokine tested. Activation of the thrombin receptor indicated that cells were fully capable of signaling.

We next assayed the ability of each of these CCR5 mutants to support fusion and infection. Both CCR5 mutants supported cell-cell fusion by the JR-FL and 89.6 Env proteins (Fig. 5B). We conclude that signaling of CCR5, as assessed by Ca^{2+} mobilization, is not required for CCR5 to function as a coreceptor. However, because cell-cell fusion and virus-cell fusion may possibly be affected differently by a GPCR signaling event, we also performed virus entry assays using a luciferase-virus packaging system previously described (16). With this assay, viruses containing the JR-FL env protein were shown to utilize both CCR5Δtail and CCR5ΔDRY (Fig. 5C). Thus, cell-cell fusion and virus-cell fusion do not require G-protein-mediated CCR5 signaling that results in Ca^{2+} mobilization.

Monoclonal antibody 12D1 recognizes CCR5. To assist in the characterization of CCR5, we have developed a monoclonal antibody, 12D1, to the amino terminus of CCR5. This antibody was produced by using a recombinant glutathione *S*-transferase fusion protein containing the first 35 amino acid residues of CCR5 and recognized native CCR5 expressed on the surface of transfected cells by flow cytometry (Fig. 6A). In addition, 12D1 detected CCR5 by immunoprecipitation (Fig. 6B) and immunofluorescence microscopy (data not shown) of transiently transfected QT6 cells. The antibody did not cross-react with CCR2b, the chemokine receptor most closely related to CCR5 (76% homology), by immunoprecipitation or flow cytometry (data not shown). Under the conditions used in this study, we have not detected any significant inhibition of cell-cell fusion with this antibody (data not shown), nor has it exhibited neutralizing activity. In addition, 12D1 appears to recognize residues on the amino terminus of CCR5, such as Asp-11, which has precluded surface quantitation of many of our mutants (data not shown). The availability of this antibody will assist future investigations into the expression patterns of CCR5.

DISCUSSION

M-tropic strains of HIV-1 that utilize CCR5 as a coreceptor are the most relevant viral strains for understanding HIV-1 transmission, as highlighted by the remarkable resistance to HIV-1 infection of individuals that lack functional CCR5 (19, 36, 41, 51). Direct interactions between Env, the chemokine receptors, and CD4 have been detected (40, 55, 59), but the mechanism by which these interactions lead to fusion is unknown. Structures of CCR5 that are important for fusion may be involved in binding Env, inducing Env into a fusogenic

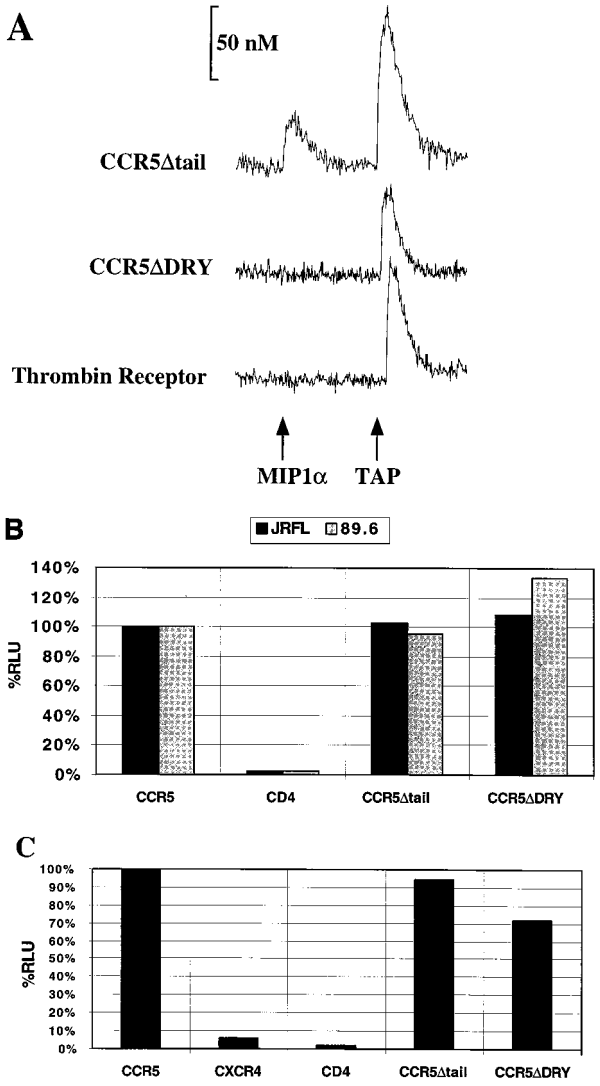

FIG. 5. (A) Independence of G-protein-mediated signaling and cofactor activity. CCR5Δtail and CCR5ΔDRY were assayed for Ca^{2+} mobilization in response to ligand MIP1α. 293T cells were transfected with CCR5Δtail, CCR5ΔDRY, or the pcDNA3 vector for 24 h, loaded with 2.5 μM Fura-2/AM, and assayed for ligand response. Cells were also cotransfected with thrombin receptor and stimulated with thrombin activating peptide 4 (TAP) 100 s after challenge with MIP1α in order to control for cell responsiveness. (B) Fusion assays were performed as described for Fig. 1 to 4, using QT6 cells as targets. The negative control CD4 received the pcDNA3 vector in place of a plasmid encoding a chemokine receptor. Results are normalized to the reporter activity measured with wild-type CCR5 (100% represents 45,344 and 36,131 relative luciferase units [RLU] for JR-FL and 89.6). (C) Luciferase virus pseudotyped with the JR-FL Env protein was used to infect U87-MG cells transfected with CD4 and the indicated coreceptors in pcDNA3. The negative control CD4 received the pcDNA3 vector in place of a plasmid encoding a chemokine receptor. Cells were harvested 4 days postinfection and assayed for luciferase activity (100% represents 1,504 RLU with a background [CD4 alone] of 113 RLU). All experiments were repeated at least three times with similar results.

conformation, or altering the subcellular environment in which fusion occurs. Identifying the structural determinants that contribute to CCR5 coreceptor function will be critical for understanding the fusion mechanism and for identifying rational targets for antiviral therapies. Our experimental strategy was designed to identify regions of CCR5 that contribute to Env-mediated fusion but does not address subtle differences between regions of CCR5 in fusion or viral entry.

Previous studies have identified the amino-terminal domain of CCR5 as sufficient for coreceptor function when placed into a highly homologous CCR2b or murine CCR5 background (5, 49). However, the amino-terminal domain of CCR5 can also be replaced by the corresponding regions from these receptors, indicating that the amino-terminal domain is not necessary and that Env-CCR5 interactions are likely complex. In this regard, both the first and second extracellular loops of CCR5 have been shown to contribute to coreceptor function. However, the consequences of CCR5 mutations on coreceptor function are also virus strain dependent. For example, the M-tropic strain JR-FL and the dual-tropic strain 89.6 exhibit differential utilization of the CCR5 amino-terminal domain (49). More strikingly, T-tropic strains of SIV that use CCR5 utilize different domains of CCR5 than M-tropic strains of SIV (26). Therefore, to critically evaluate the structural determinants of CCR5 coreceptor function, we placed mutations in multiple contexts and examined their effects on coreceptor function for envelopes of multiple virus strains.

In agreement with previous results (5), we found that the murine CCR5 homolog (82% identity) did not support fusion by any HIV-1 Env tested, including 89.6 Env. The ability of 89.6 to utilize chemokine receptors that are far more divergent from human CCR5 than is the murine homolog suggests that murine CCR5 lacks one or more critical residues that are required for coreceptor activity. Examination of the murine sequence identifies 22 extracellular residues that differ from human CCR5, 10 of which are present in the amino-terminal domain and 11 of which are present in the first and second extracellular loops (Fig. 7). Murine and human CCR5 differ by only a single amino acid in the third extracellular loop, and analyses of chimeras between the two receptors, as well as between CCR2b and CCR5, have not been able to determine the importance of this conserved region for virus fusion (5, 49).

The number of amino acid differences between human and murine CCR5 in the first three extracellular domains, particularly the amino-terminal region, prompted us to construct a series of chimeras between CCR5 and more highly divergent chemokine receptors in order to more rigorously assess the functional roles of these domains and the degree of interdependence between them. Consistent with earlier work, we found that the amino-terminal domain of CCR5 plays a major role in Env-mediated fusion by conferring coreceptor function to CCR2b (76% homology with CCR5), CCR1 (56%), CXCR2 (34%), and CXCR4 (33%) for JR-FL and 89.6 envelopes. The reciprocal chimeras confirmed the importance of the amino terminus for the dual-tropic virus 89.6 but also demonstrated the functional independence of the loops of CCR5. While chimera 1555 supported cell-cell fusion by both JR-FL and 89.6, chimeras 2555, B555, and L555 did not support fusion by 89.6. Thus, M-tropic envelopes can tolerate dramatic substitutions of either the amino-terminal domain or the extracellular loops of CCR5, suggesting the existence of two distinct structures on CCR5, either of which can support Env-mediated cell-cell fusion. Indeed, JR-FL and other M-tropic viruses continue to utilize a mutant of CCR5 that lacks a large portion (16 amino acids) of its amino terminus (49). In contrast, dual-tropic Env proteins like 89.6 and RF are highly dependent on the amino-terminal domain of CCR5. The hypothesis of two sites on CCR5 through which Env can interact has precedent in the natural binding pattern of chemokine receptors and their ligands. The chemokine receptors IL8-RB and CCR2b and the C5a chemoattractant receptor share a two-step mechanism of ligand binding in which the amino terminus and the loops of the receptor mediate independent interactions with ligands (1, 21, 35, 44, 54).

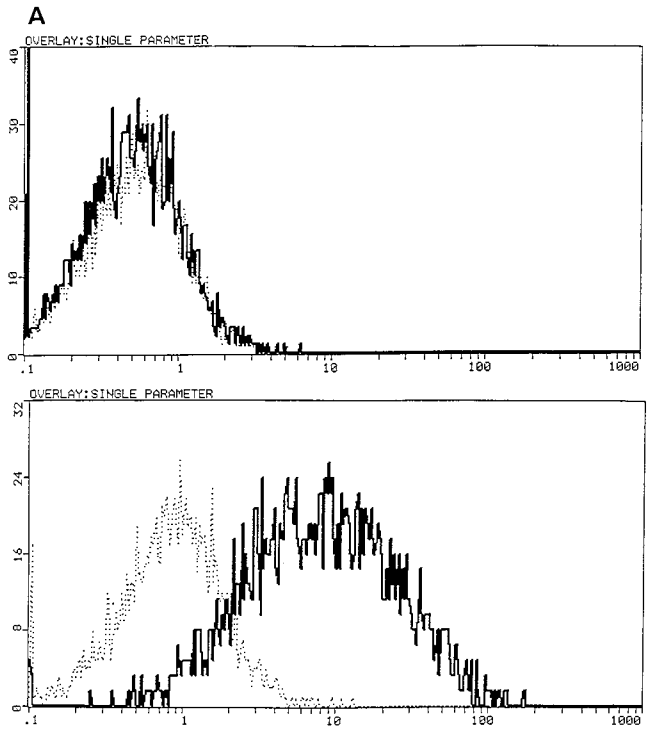

FIG. 6. Recognition of CCR5 by monoclonal antibody 12D1. (A) Flow cytometric analysis of QT6 cells transiently transfected with CCR5 (bottom) or mock transfected with pcDNA3 vector (top). Cells were stained with monoclonal antibody 12D1 (black) or mouse IgG (gray). (B) Immunoprecipitation of CCR5. QT6 cells were prepared to express either CCR5-HA or a control vector. Lysates of the cells were prepared and immunoprecipitated with 12CA5 (recognizes the HA tag), 12D1 (recognizes CCR5), or D47 (a control monoclonal antibody that recognizes HIV gp120). Samples were run on SDS–10% polyacrylamide-urea gels and probed with the 12CA5 antibody. The arrow indicates the predicted molecular mass of CCR5 (41 kDa with the HA tag). Left and right panels are from independent experiments.

Since charged amino acids in the V3 loop of envelope proteins are known to make significant contributions to Env tropism (13, 20, 29, 38), and since the V3 region makes a major contribution to coreceptor specificity (14), we reasoned that charged residues in the extracellular domains of CCR5 may be critically involved in the fusion mechanism and/or Env specificity. One such residue, Asp-11, appears to play a central role for several important Envs. Asp-11 is required by the dual-tropic RF Env, is central to the structure required for fusion by the dual-tropic primary isolate 89.6, and is very important for fusion by the divergent clade E primary isolate CM243. The importance of Asp-11 was implicated in our previous study using amino-terminal truncations of CCR5 in which residues 10 to 13 were shown to be important for fusion by 89.6 but were not required by JR-FL (49). The implication that multiple structures are involved in fusion prompted us to create double- and triple-mutant CCR5 molecules. While many of these mutants demonstrated no effect on cell-cell fusion (Fig. 3 and data not shown), our results demonstrate that residues in the amino-terminal domain (Asp-11), the second extracellular loop (Lys-197), and the third extracellular loop (Asp-276) contribute to a conformationally complex structure required by 89.6 Env. Asp-11 appears to be central to this structure, since mutation of Lys-197 and Asp-276 does not affect coreceptor activity for 89.6. It is also intriguing that CCR1, but not CCR2b, CXCR2, or CXCR4, contains an aspartic acid at position 11, possibly explaining why 89.6 is able to use the 1555 chimera but not the 2555, B555, or L555 chimera. The residues identified here, however, are clearly not the only amino acids involved in fusion and Env specificity. Asp-11, Lys-197, and Asp-276 are present in the nonfusogenic mouse CCR5 homolog, and mutation of the three residues does not eliminate fusion by all viral strains.

The evidence for two distinct sites on CCR5 that interact with Env offers a mechanism for the evolution of chemokine receptor usage in vivo. Viral isolates that use CCR5 can, over time, acquire the ability to utilize divergent chemokine receptors such as CXCR4 (18). However, the ability of a virus to utilize two different coreceptors may limit the ways in which the virus can individually utilize each coreceptor. Thus, the dual-tropic Envs that we have studied, those from 89.6 and RF, are highly sensitive to changes in chemokine receptor structure, particularly in the amino-terminal domain. This dependence on the amino terminus of CCR5 may indicate that the evolution to dual tropism proceeds by changes in Env that enable it to use relatively conserved regions of the chemokine receptors, such as the extracellular loops, while retaining the ability to use the more highly divergent amino-terminal domain of CCR5, thus bridging the transition from M tropism to dual tropism. Indeed, we have recently found that the first and second extracellular loops of CXCR4 are sufficient for coreceptor function for most virus strains tested, including 89.6 (42). Thus, the ability of 89.6 to use both CCR5 and CXCR4 is due, in part, to its utilization of distinct, nonoverlapping domains within each of these receptors.

Receptor signaling and internalization have the potential to influence the subcellular location and environment where fusion occurs. Such a signal could also affect postentry steps in viral replication that may provide insight into the mechanism by which the chemokine receptors function as viral cofactors. To directly address the importance of signal transduction on coreceptor function, we constructed cytoplasmic domain mutants designed to eliminate the signal transduction capability of CCR5. Mutation of the highly conserved DRY motif located in the second intracellular loop of CCR5 eliminated induction of a Ca^{2+} flux in response to ligand binding. Similar mutations have been shown to eliminate G-protein coupling and signaling by other GPCRs, such as rhodopsin and the α- and β-adrenergic receptors (30–32, 58), and recent reports have confirmed that CCR5 signaling is not required for viral infection (27, 33). In contrast, elimination of nearly all Ser and Thr residues from the cytoplasmic domains of CCR5 was not sufficient to eliminate chemokine-induced signaling. Nevertheless, the ability of CCR5ΔDRY to support both cell-cell and virus-cell fusion indicated that a downstream signal from CCR5 was not required for either event to occur. Much like its use of CD4, whose cytoplasmic domains are not required for entry (6, 23), HIV-1 appears to have adapted itself to use the chemokine receptors for fusion independent of their normal functions.

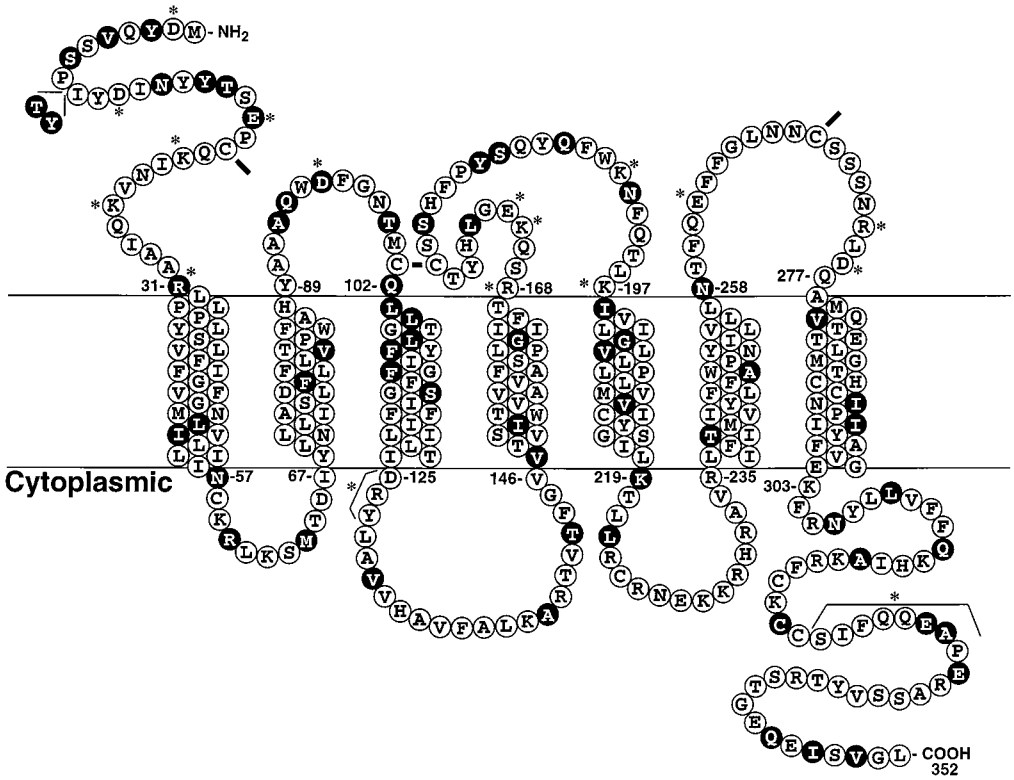

FIG. 7. Structural features of CCR5. A representation of the human CCR5 molecule is shown. Shaded residues indicate human residues that are different from those in the mouse CCR5 homolog. The TY residues inserted between residues 8 and 9 represent amino acids that are present in murine CCR5 but are not present in human CCR5. Bars at Cys residues represent potential Cys bonds formed between the first and second loops and between the amino-terminal domain and third extracellular loop. Asterisks highlight mutations of CCR5 used in this study. The cytoplasmic deletion present in CCR5Δtail is indicated by an asterisk and bar encompassing the deleted region.

Although HIV-1 did not require G-protein-mediated signaling of CCR5 for fusion, chemokine receptor signals may still mediate virus-induced effects secondary to fusion. For example, the T-tropic virus SIVmac239 is able to use CCR5 for fusion and is able to enter macrophages but is unable to replicate in this cell type (45). Localization of this postentry block to the Env protein of SIVmac239 offers the intriguing possibility that activation of a chemokine receptor by some viruses influences postentry steps of infection in some nonmitotic cell types such as macrophages. Consistent with this hypothesis, we have previously observed differential utilization of CCR5 by M- and T-tropic strains of SIV (26). Most recently, we have tested all of the chimeras presented here and some of our most significant point mutants by infection assays. While all of these mutants support entry of one or more HIV-1 strains, differences between cell-cell fusion and productive virus infection are sometimes observed, depending on both the cell type and the virus strain used (unpublished data). We are currently characterizing these differences, as they may differentiate Env-mediated fusion from productive entry of HIV, perhaps reflecting potential postentry effects due to differential CCR5 utilization as has been recently suggested (12). Although CCR5 signaling did not influence the ability of CCR5 to act as a fusion coreceptor, the ability of CCR5 to influence postentry steps of infection in some virus-cell type combinations remains to be investigated.

The studies presented here provide a more detailed picture of the structural determinants that are required for CCR5 coreceptor function and provide compelling evidence that Env interacts with CCR5 via multiple, distinct regions of the chemokine receptor. The ability of multiple CCR5 domains to participate in the fusion mechanism was demonstrated by the observations that no single charged residue in the CCR5 extracellular domains was required for coreceptor function, that no single CCR5 domain was required for coreceptor function for most Env proteins, and that viruses like 89.6 can use multiple chemokine receptors with highly divergent ectodomain sequences. In addition, the identification of specific residues in distinct CCR5 domains that are simultaneously required by 89.6 suggests that these residues participate in the formation of a complex structure that interacts with Env. The role that each of these domains plays in Env binding, conformational change induction, and possible signal transduction events may vary between Env proteins of different strains. Furthermore, the existence of two distinct CCR5 domains that can impart coreceptor function to diverse chemokine receptor backgrounds suggests the existence of at least two complementary sites on Env, one of which can specify the interaction with CCR5 while the other is more free to diverge and specify interactions with other chemokine receptors. Precedent for this model has been demonstrated for the chemokine-related C5a chemoattractant in which independent binding sites on the receptor interact with two different sites on the C5a ligand (21). The analysis of a much larger panel of virus isolates will be required to characterize the structural features in both Env and CCR5 that complement one another and lead to membrane fusion.

ACKNOWLEDGMENTS

Benjamin J. Doranz and Zhao-hai Lu contributed equally to this work.

We thank Mike Endres and Joanne Berson for their contributions to this work. We also thank Aimee Edinger, Jim Hoxie, Richard Horuk, Joe Hesselgesser, Mike Orsini, Peter O'Brien, Lawrence Brass, Chris Broder, and Marc Parmentier for discussions and advice. Harley Jenks, Chris Worth, and Tina Sung provided excellent technical assistance throughout this project. We thank Bob Andrews for primate samples. A number of important reagents were obtained from the NIH AIDS Research and Reference Reagent Program.

S.C.P. was supported by Agnes Brown Duggan and Humana endowments. R.W.D. was supported by NIH grants AI-35383 and AI-38225. B.J.D. was supported by a Howard Hughes Medical Institute predoctoral fellowship.

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