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Multiple Extracellular Domains of CCR-5 Contribute to Human Immunodeficiency Virus Type 1 Entry and Fusion

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Human immunodeficiency virus type 1 (HIV-1) entry is governed by the interaction of the viral envelope glycoprotein (Env) with its receptor. The HIV-1 receptor is composed of two molecules, the CD4 binding receptor and a coreceptor. The seven-membrane-spanning chemokine receptor CCR-5 is one of the coreceptors used by primary isolates of HIV-1. We demonstrate that the mouse homolog of CCR-5 (mCCR-5) does not function as an HIV-1 coreceptor. A set of chimeras of human CCR-5 and mCCR-5 was studied for Env-induced cell fusion and HIV-1 infection. Using the HIV-1_{ADA} envelope glycoprotein in a syncytium formation assay, we show that replacement of any fragment containing extracellular domains of mCCR-5 by its human counterparts is sufficient to allow Env-induced fusion. Conversely, replacement of any fragment containing human extracellular domains by its murine counterpart did not lead to coreceptor function loss. These results show that several domains of CCR-5 participate in coreceptor function. In addition, using a panel of primary nonsyncytium-inducing and syncytium-inducing isolates that use CCR-5 or both CXCR-4 and CCR-5 as coreceptors, we show that the latter dual-tropic isolates are less tolerant to changes in CCR-5 than strains with a more restricted coreceptor use. Thus, different strains are likely to have different ways of interacting with the CCR-5 coreceptor.

Human immunodeficiency virus type 1 (HIV-1) enters target cells by fusion of its envelope with the membrane of the cell. This process is initiated by the high-affinity binding of the envelope glycoproteins (Env and gp120) to the CD4 molecule. Subsequent to the Env-CD4 interaction, a number of conformational changes in gp120 and probably in CD4 take place, leading to virus entry or syncytium formation (reviewed in references 58 and 83). These post-CD4 binding events are not completed in most nonhuman cell lines and in some human cell lines expressing human CD4, and membrane fusion fails to occur (5, 20, 23, 53). It was suggested that these cells lack a component necessary for the fusion and entry processes to be completed (13, 35, 41). Many of these experiments were done with syncytium-inducing (SI) T-cell-line-adapted (TCLA) strains of HIV-1, but later evidence suggested that different molecules may be required for the entry of primary non-SI (NSI) isolates (2, 12). The second components of the receptor complex, or coreceptors, have been identified (3, 22, 32, 34, 36, 38) and belong to the seven-transmembrane G protein-coupled chemokine receptor family (reviewed in reference 69).

As predicted, the coreceptors for TCLA isolates and primary isolates are different: the CXCR-4 chemokine receptor (previously known as LCR1, LESTR, HUMSTR, or fusin) functions as an HIV-1 coreceptor for TCLA as well as for primary SI or T-cell-tropic strains, whereas all primary NSI or macrophage-tropic (M-tropic) strains tested so far use the CCR-5 chemokine receptor (3, 22, 32, 34, 36, 38, 78). Other members of the chemokine receptor family, including CCR-2b and CCR-3, can also be used by a restricted number of HIV-1 isolates (22, 34, 42, 71). This extension of the use of the coreceptor by primary isolates has been shown to correlate with disease progression (30). However, CXCR-4 and CCR-5 seem to be the main coreceptors used by HIV-1 in vivo, irrespective of the viral genetic subtype (89). The importance of CCR-5 as an HIV-1 coreceptor in vivo has been confirmed with the identification of a *CCR-5*-defective allele in some multiply exposed uninfected individuals (31, 43, 51, 73).

Chemokines are small proteins involved in cellular recruitment and activation. They show some sequence homology and can be divided into four subgroups according to the spacing of their amino-terminal cysteine residues. The two main groups are CXC (or α) chemokines and CC (or β) chemokines (reviewed in references 69 and 84). CXCR-4 binds the CXC chemokine stromal cell-derived factor 1 (9, 60), and CCR-5 binds RANTES, macrophage inflammatory protein 1α (MIP-1α), and MIP-1β (29, 70, 72), which are CC chemokines. These chemokine ligands are able to block HIV-1 entry and fusion (9, 26, 27, 60, 63, 65). In addition, receptor antagonists based on these chemokines can also block HIV-1 entry (4, 76). The exact mechanism of this inhibition is not yet known but most likely involves steric blockade of the coreceptor by the ligand. Alternatively, chemokine-mediated inhibition of HIV entry may be due to down-regulation of the receptor on the surfaces of permissive cells.

In fact, it has recently been suggested that a ternary complex consisting of gp120, CD4, and the coreceptor forms on the surfaces of permissive cells (50, 81, 88). The molecular details of the interaction between gp120, CD4, and the coreceptor are still not clear. It has been demonstrated that the third hypervariable region (V3 loop) of the gp120 envelope glycoprotein is likely to be a component of the coreceptor binding site (81, 88) and to influence coreceptor choice (22, 33, 67), but the interaction between gp120 and the coreceptor molecule has not yet

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been completely characterized. Using molecular chimeras composed of two members of the CC chemokine receptor family, CCR-5 and CCR-2b (6, 71), or of mouse and human CCR-5 (mCCR-5 and hCCR-5, respectively) (6, 8), three groups of researchers have recently shown that the amino-terminal and possibly the first and second extracellular loops of CCR-5 are important for coreceptor function and specificity. Two of these studies, however, assessed coreceptor use by cell-cell fusion only (8, 71), while the third (6) used cell-free virus infection, but with a limited number of isolates only. Although cell-cell fusion usually correlates well with cell-free virus infection, some exceptions to this rule have been reported (55, 77).

To map the regions important for CCR-5 to function as an HIV-1 coreceptor for cell-cell fusion and virion-cell fusion, we took advantage of the existence of the mouse homolog of CCR-5 (mCCR-5) (10, 57). We confirm here that this molecule does not function as a coreceptor for HIV-1. By generating molecular chimeras composed of hCCR-5 and mCCR-5, we have further defined regions in the coreceptor that are important for HIV-1 entry and fusion with Env-expressing cells. Several domains of CCR-5 are shown to be involved in HIV-1 entry. Moreover, different HIV-1 isolates showed various patterns of interaction, implying a differential use of the CCR-5 coreceptor for NSI M-tropic versus SI dual-tropic strains.

MATERIALS AND METHODS

Cells and viruses. CCC-CD4 cells are cat kidney cells stably transfected with a human CD4 expression vector (24). HeLa-CD4-LTR*lacZ* (HeLa-P4) cells are HeLa cervical epithelial cells stably transfected with human CD4 and a long terminal repeat (LTR)-*lacZ* construct (25). Upon infection by HIV or fusion with Tat-expressing cells, transcription of the *lacZ* gene directed by the LTR is stimulated and leads to the accumulation of β-galactosidase in the cell. HeLa-Env cells are HeLa cells stably transfected with a Δ*gag-pol* provirus containing a *dhfr* gene in place of *nef*, which confers resistance to methotrexate. HeLa-Env_{LAI} (74) and HeLa-Env_{ADA} (a gift from A. Brelot, Institut Cochin de Génétique Moleculaire, Paris, France) (68) respectively express the HIV-1 LAI and ADA envelope glycoproteins. All these HeLa-Env cells also constitutively express the HIV-1 transactivator Tat. All the adherent cell lines were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (FCS) (or 5% FCS for CCC-CD4), antibiotics, and 0.5 mg of G418 (CCC-CD4 and HeLa-P4) per ml or 5 μM methotrexate (HeLa-Env).

All the HIV-1 isolates used in this study have been described previously. LAI (82) is a TCLA strain, and GUN-1 (80) is also TCLA but dual tropic, as it can also infect macrophages (56, 77). 89.6, a gift from R. Collman (University of Pennsylvania, Philadelphia), is a primary HIV-1 strain that infects macrophages and certain $CD4^+$ T-cell lines and is therefore also dual tropic (28). ADA (86), SF-162 (19), M23, and E80 are primary NSI M-tropic strains of HIV-1 (78). All isolates are of subtype B except E80 and M23, which are unclassified. Viral stocks from primary isolates and TCLA strains were produced in peripheral blood mononuclear cells (PBMCs) and H9 cells, respectively, as described previously (78). ADA viral stocks were produced by transfection of the molecular clone in HeLa cells (1). The ADA-producing plasmid, a gift from M. Alizon (Institut Cochin de Génétique Moléculaire, Paris, France), is a recombinant between the 90.1 molecular clone of $HIV\text{-}1_{LAI}$ and the *Kpn*I-*Ava*I fragment encompassing the ADA envelope protein (68).

hCCR-5 and mCCR-5 chimera construction. The hCCR-5 (70) (a gift from P. Gray, ICOS Corp., Bothell, Wash.), mCCR-5 (57), and CXCR-4 cDNAs were subcloned by PCR into the pcDNA3 expression vector (Invitrogen, Leek, The Netherlands) between the *Hin*dIII and *Not*I sites. The sequences, determined by automated sequencing (Applied Biosystems) with a Perkin-Elmer sequencing kit and analyzed with the Genetics Computer Group package, were found to differ at a few positions from the published sequences (Fig. 1). The differences resulted in conservative amino acid (aa) changes: F80L in mCCR-5 and F166L in hCCR-5. The cDNAs encoding the receptors were fused at the carboxy-terminus to the 12-aa-long c-*myc* epitope tag: NH_2-GGREQKLISEEDLA-COOH (37). We decided to tag all the constructs at the end of the C-terminal intracellular domain to avoid interference with potential interactions between CD4 and/or gp120 and extracellular segments of the coreceptor. To generate hCCR-5 and mCCR-5 chimeras, a unique *Eco*RV restriction site was engineered by site-directed mutagenesis (Transformer kit; Clontech, Palo Alto, Calif.) in the hCCR-5 sequence at the same position as in the mCCR-5 sequence (Fig. 1). Using the same technique, we also introduced a unique *Kpn*I restriction site in the mCCR-5 sequence at the same position as in the hCCR-5 sequence (Fig. 1). Introduction of these new common sites did not introduce changes in the amino acid sequences of the proteins. The different chimeric constructs were then generated by exchanging fragments by using the four common unique restriction sites, *Hin*dIII, *Eco*RV, *Kpn*I, and *Not*I. The *Hin*dIII-*Eco*RV fragment spans the N terminus, transmembrane 1 (TM1), and intracellular loop 1 (I1) of CCR-5; the *Eco*RV-*Kpn*I fragment spans TM2, extracellular loop 1 (E1), and TM3; and the *Kpn*I-*Not*I fragment spans I2, TM4, E2, TM5, I3, TM6, E3, TM7, and the C terminus. The structures of the hCCR-5–mCCR-5 chimeras are schematically depicted in Fig. 2.

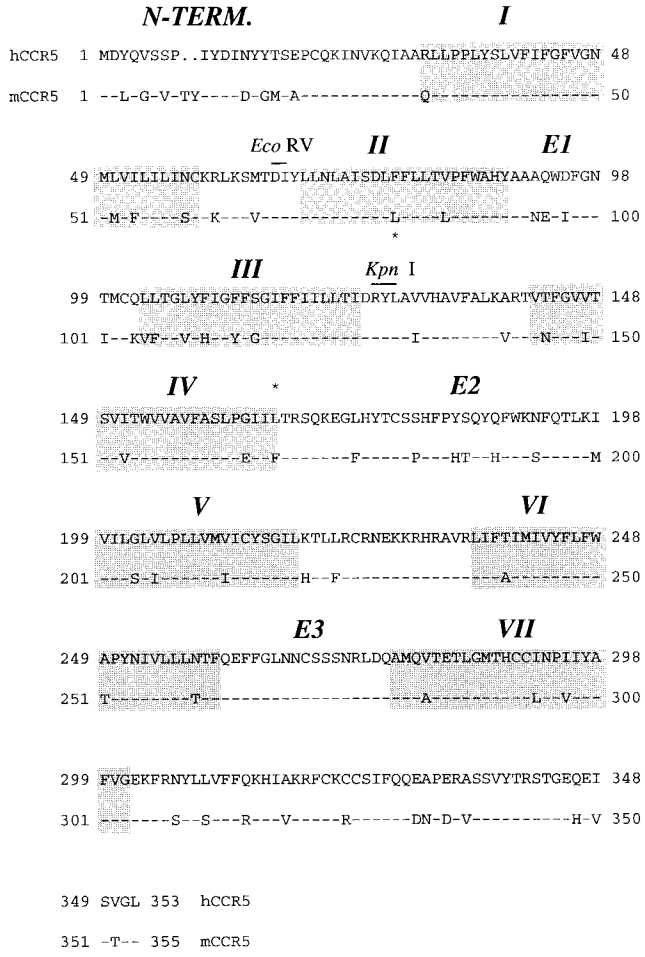

FIG. 1. Amino acid sequence alignment of hCCR-5 and mCCR-5. The two sequences were aligned with the gap program. The sequences display 91% similarity and 83% identity. Potential transmembrane segments (listed under SwissProt accession no. P51682) are shaded and numbered with Roman numerals. Extracellular loops are also numbered. Restriction endonuclease sites are labeled and overlined. Only changes at the amino acid level are shown, while identical amino acids are indicated by dashes. Asterisks identify differences from the published sequences. N-TERM., N terminus.

CCR-5 cell surface expression. The constructs encoding the different CCR-5 chimeras were transfected into subconfluent HeLa-P4 or CCC-CD4 cells (5 μg per 50-mm-diameter petri dish) by the calcium phosphate precipitation method. After 16 to 20 h of incubation, the cells were washed in phosphate-buffered saline (PBS), fed, and incubated for another 48 h. The cells were then fixed in methanol-acetone (1:1) for 5 min at room temperature and subsequently washed in PBS–1% FCS. The cells were then incubated with purified anti-*myc* (9E10) monoclonal antibody (1:100 in PBS–1% FCS) for 1 h at room temperature. After four washes in PBS–1% FCS, fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G1 antibody (Southern Technologies Associates Inc., Birmingham, Ala.) was added for 1 h at room temperature (1:100 in PBS–1% FCS). After two washes in PBS–1% FCS and two washes in PBS, the cells were mounted in Mowiol (Calbiochem) and observed on a Zeiss fluorescence microscope. Surface expression was estimated by randomly counting 10 fields. Cells were scored as positive if the signal was associated with the cell plasma membrane.

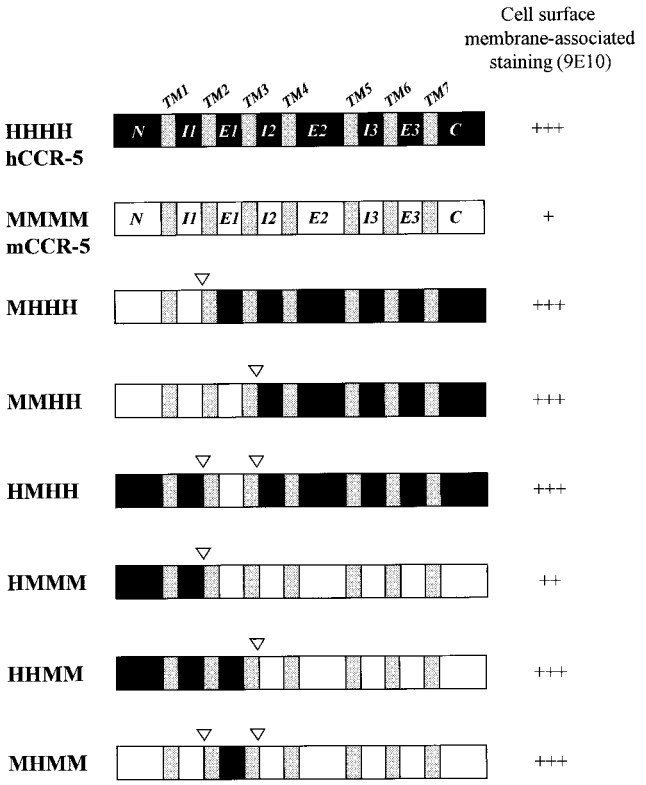

FIG. 2. Schematic representation of hCCR-5–mCCR-5 chimeras. Segments of human origin are filled, while segments of murine origin are open. Transmembrane domains are shaded, and inverted triangles indicate junction sites. The relative level of surface membrane-associated expression of each chimeric receptor, as estimated by immunofluorescence 2 to 3 days after transfection of HeLa-P4 or CCC-CD4 cells (see Materials and Methods), is indicated on the right-hand side and expressed as the percentage of hCCR-5 expression as follows: +, 20 to 50%; ++, 50 to 70%; and +++, 70 to 100%. These estimations are representative of three independent experiments.

Fusion assay. HeLa-P4 cells were plated in 12-well (or 24-well) trays and transfected by the calcium phosphate precipitation method the next day with the different CCR-5 constructs with 1 (or 0.5) μg of DNA per well. After 16 to 20 h, the cells were washed in PBS, split into four replicates in 96-well plates, and cocultivated with HeLa-Env_{ADA} or HeLa-Env_{LAI} (10^4 cells/well) for 24 h. An in situ X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) assay was then done in duplicate. In brief, cells were washed in PBS, fixed in PBS–0.5% glutaraldehyde for 5 min, washed in PBS, and incubated with the X-Gal substrate (0.5 mg of X-Gal per ml in PBS containing 3 mM ferricyanide, 3 mM ferrocyanide, and 2 mM $MgCl_2$; Calbiochem) for 2 h at 37°C. The two other wells were assayed for β-galactosidase production by a colorimetric assay (59). After being washed in PBS, the cells were lysed in 100 μl of PBS–0.5% Nonidet P-40 for 5 min at room temperature. Fifty microliters of that lysate was then mixed with 50 μl of 2× CPRG substrate (16 mM chlorophenol red–β-D-galactopyranoside [Boehringer Mannheim], 0.12 M $Na_2HPO_4 \cdot 7H_2O$, 0.08 M $NaH_2PO_4 \cdot H_2O$, 0.02 M KCl, 0.02 M $MgSO_4 \cdot 7H_2O$, 0.01 M β-mercaptoethanol) in a 96-well plate and incubated for 2 to 3 h in the dark at room temperature. These conditions were checked for linearity of the enzymatic colorimetric reaction. The absorbance at 540 nm was then read on a Dynatech microplate reader.

Infectivity assay. Subconfluent CCC-CD4 cells in 100 or 50-mm-diameter petri dishes were transfected with 10 or 5 μg of the different CCR-5 constructs by using Lipofectamine (Gibco BRL, Life Technologies) or calcium phosphate precipitation for 5 or 16 to 20 h, respectively. The cells were then split into 48-well plates at 2×10^4 to 4×10^4 cells/well. They were challenged the next day with HIV-1 ($\sim 10^3$ to 10^4 focus forming units per well) for 2 to 5 h, refed, immunostained 3 days postinfection with an anti-p24 antibody as the primary antibody, and subsequently incubated with a secondary antibody conjugated to β-galactosidase (Genosys Biotechnologies, Inc.) as described previously (24). The blue-stained syncytia were then scored after an in situ X-Gal assay.

RESULTS

The murine homolog of CCR-5 is inactive as a coreceptor for HIV-1. The murine homolog of CCR-5 (mCCR-5) was recently cloned and has 83% identity and 91% similarity at the amino acid level to hCCR-5 (57). The differences are not located in a particular region of the molecule but are distributed throughout the sequence (Fig. 1). The murine protein is a structural and functional homolog of hCCR-5, since it acts as a receptor for mouse MIP-1α, mouse MIP-1β, and mouse RANTES (10, 57). Because of its closely related sequence and chemokine binding profile, we decided to test its functionality as an HIV-1 coreceptor.

hCCR-5 and mCCR-5 were tagged at their C termini with the c-*myc* epitope (9E10), cloned into a pcDNA3 expression vector, and transfected into HeLa-P4 cells. This $CD4^+$ HeLa cell line is stably transfected with a *lacZ* gene under the control of the HIV-1 LTR and is therefore inducible by the HIV-1 transactivator Tat. Because of their human origin and the endogenous expression of the CXCR-4 receptor (38), HeLa-P4 cells are permissive for entry and fusion of TCLA and primary SI HIV-1 strains but not for primary NSI M-tropic strains. After transfection of hCCR-5 or mCCR-5 into HeLa-P4 cells, the cells were cocultivated with HeLa-Env_{LAI} or HeLa-Env_{ADA} cells. As expected, HeLa-Env_{LAI} were able to form syncytia with untransfected cells as well as with hCCR-5- and mCCR-5-transfected cells (not shown). HeLa-Env_{ADA}, however, could form syncytia only with HeLa-P4 cells transfected with hCCR-5, as mCCR-5 did not serve as a coreceptor for HIV-1_{ADA} Env-induced cell fusion (Fig. 3 and 4). The surface expression level of the mCCR-5 protein in HeLa-P4 cells was fivefold less that of hCCR-5 (Fig. 2). However, overexpression of mCCR-5 in $CD4^+$ CCC cat kidney cells or 3T3 mouse fibroblasts by vaccinia virus/T7 RNA polymerase infection did not result in syncytium formation upon cocultivation with HeLa-Env_{ADA} while hCCR-5 did (data not shown). Thus, despite its similarity with hCCR-5, mCCR-5 is not functional as an HIV-1 coreceptor. We therefore used this property to map the regions of CCR-5 important for HIV-1 entry and fusion by using chimeric CCR-5 receptors.

Mapping of CCR-5 extracellular domains involved in HIV-1_{ADA} envelope-induced fusion. To map the extracellular domains involved in coreceptor function for HIV-1 NSI M-tropic strains, we chose to use the ADA isolate as an Env prototype. Since hCCR-5 and mCCR-5 are conserved at the amino acid level, it was possible to construct functional molecular chimeras between the two receptors (described in Materials and Methods). The nomenclature that we used for the different regions of chemokine receptors is as follows: E1, E2, and E3 for extracellular loops 1 to 3, amino-terminal extracellular domain, I1, I2, and I3 for intracellular loops 1 to 3, carboxy-terminal intracellular domain, and TM1 to TM7 for the seven-membrane-spanning segments. We reasoned that to be used as an HIV-1 coreceptor, the important functional domains of the molecule were likely to be the four extracellular domains (N terminus, E1, E2, and E3). However, we should bear in mind that the transmembrane and intracellular domains can play a role in the overall conformation of the receptor, in the interaction with gp120 and/or CD4, or in the fusion step per se. There are seven amino acid differences (and a 2-aa insertion in mCCR-5) between hCCR-5 and mCCR-5 amino-terminal extracellular domains (30 to 32 aa long), five differences in E1 (13 aa long), seven differences in E2 (32 aa long), and no differences in E3 (17 aa long). We generated six chimeras by exchanging fragments encompassing each of the first three extracellular domains. The resulting constructs are depicted in

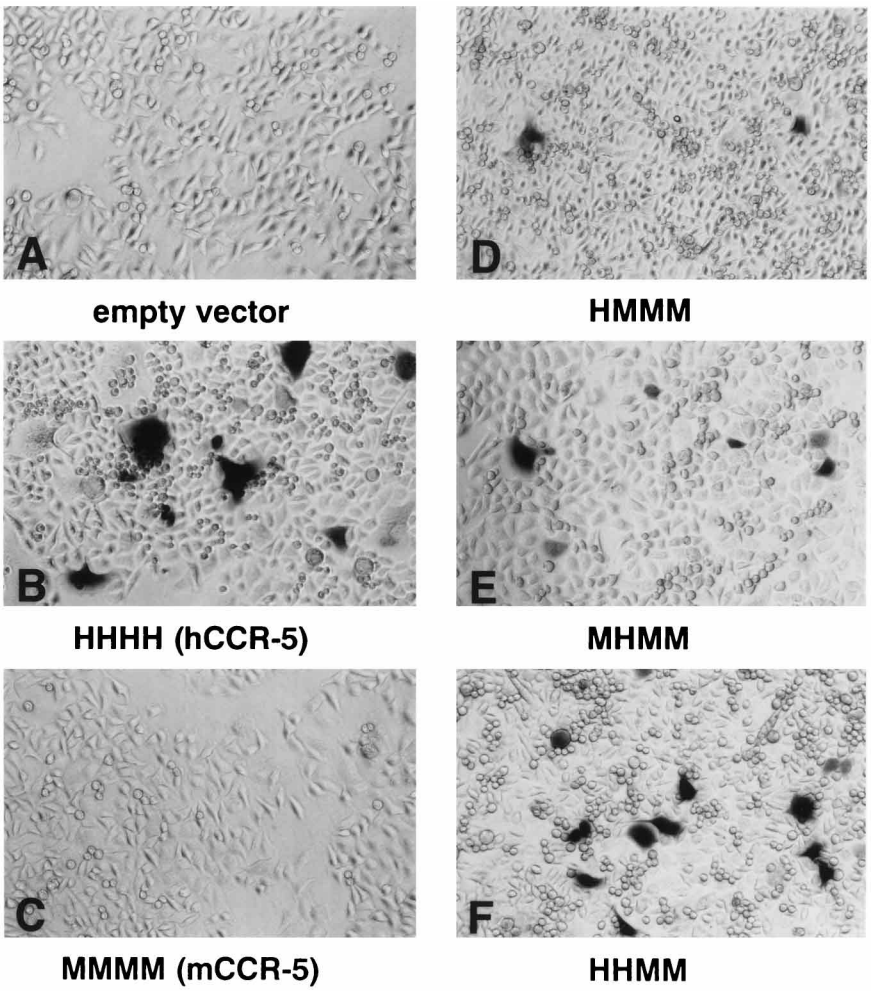

FIG. 3. Formation of syncytia between HIV-1_{ADA} envelope-expressing cells and HeLa-P4 cells expressing different hCCR-5–mCCR-5 chimeras. HeLa-P4 cells transiently transfected with an empty vector (A), hCCR-5 (B), mCCR-5 (C), or the different chimeras HMMM (D), MHMM (E), and HHMM (F) were cocultivated for 24 h with HeLa-Env_{ADA} cells, fixed, and stained with the X-Gal substrate as described in Materials and Methods. Photographs were taken at a ×160 magnification. Results to similar those seen in panels B and F were obtained for the MHHH, HMHH, and MMHH chimeric receptors.

Fig. 2. Each of the four extracellular domains is represented by the letter H or M depending on the human or murine origin of the extracellular domains in the chimera, and the constructs are named accordingly.

Levels of cell surface expression of the six chimeras and the two parental clones were estimated by transfecting the C-terminus-tagged constructs into HeLa-P4 or CCC-CD4 cells and staining them with the 9E10 (c-*myc* epitope) monoclonal antibody by indirect immunofluorescence after permeabilization of the cells. Cells were scored as positive if the 9E10 staining was associated with the cell plasma membrane. As previously mentioned, mCCR-5 was expressed at a lower level than its human counterpart. All other hCCR-5–mCCR-5 constructs were expressed at levels similar to that of the hCCR-5 parental clone except for the HMMM chimera, which showed about 50% of the level of hCCR-5 expression (Fig. 2).

These different chimeras were tested for coreceptor activity by transient transfection into HeLa-P4 cells and coculture with HeLa-Env_{ADA}. Blue-stained syncytia were scored after an in situ X-Gal assay, or β-galactosidase activity was measured by a CPRG colorimetric assay (Fig. 3 and 4). To map the domain(s) necessary and sufficient to confer coreceptor activity to mCCR-5, we first introduced segments of hCCR-5 containing each extracellular domain into nonfunctional mCCR-5. The HMMM chimera inefficiently mediated HIV-1_{ADA} Env-induced fusion (Fig. 3 and 4), indicating that a fragment encompassing the N-terminal, TM1, and I1 domains was not sufficient to confer complete coreceptor activity or that the lower level of expression of this construct resulted in reduced coreceptor activity. However, when we introduced a fragment containing the TM1, E1, and TM2 domains in mCCR-5 (MHMM), it was possible to observe syncytium formation with Env-expressing cells. This fusion activity was reproductively about 30% of the level of the hCCR-5 control when we assayed by the CPRG colorimetric assay (Fig. 4). This reduced activity was due to a reduction in the size of syncytia (Fig. 3). Thus, the first extracellular loop when it is introduced into the mCCR-5 receptor is sufficient to mediate ADA Env-mediated fusion. To determine the role of the second extracellular loop, we used the MMHH chimera, which is identical in extracellular sequence to the theoretical MMHM chimera. This chimera had coreceptor activity similar to that of parental hCCR-5, indicating that E2 is also important in mediating HIV-1 Env interaction.

We then placed two domains of hCCR-5 into the nonfunc-

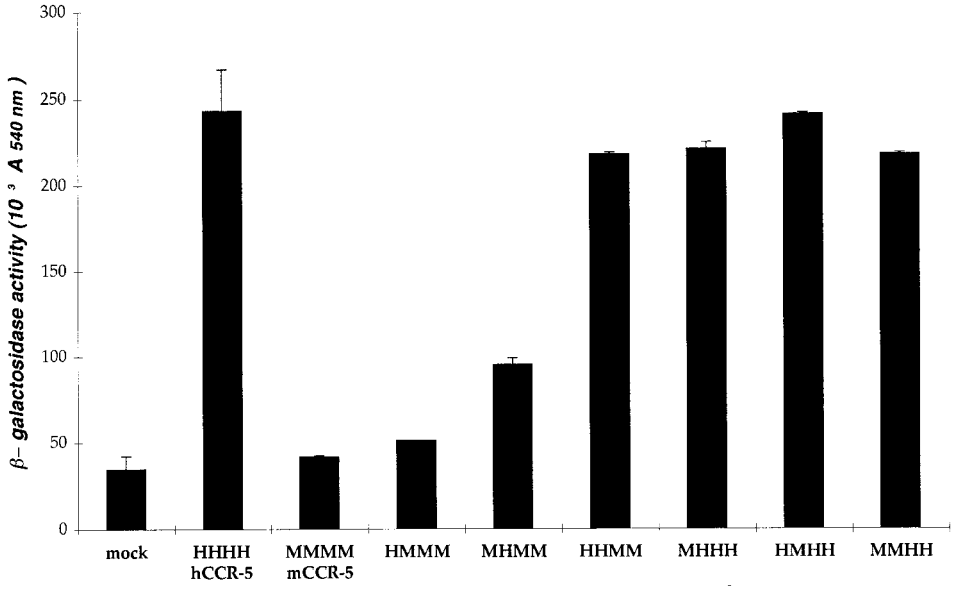

FIG. 4. Fusion assay between HIV-1_{ADA} envelope-expressing cells and HeLa-P4 cells expressing different hCCR-5–mCCR-5 chimeras. HeLa-P4 cells transiently transfected with the different constructs were cocultivated for 24 h with HeLa-Env_{ADA} cells in 96-well plates, lysed, and tested for β-galactosidase activity by a colorimetric CPRG assay as described in Materials and Methods. The histogram presented here is representative of three independent experiments done in duplicate. The results are expressed as the means of A_{540} values ± standard deviations.

tional murine background. The chimera HHMM (for MMHH, see above) worked as well as the hCCR-5 parental molecule for ADA-induced fusion (Fig. 3 and 4) and better than the first two individual extracellular domains alone (see HMMM and MHMM). This construct confirms that the first two extracellular domains are important for coreceptor activity. Furthermore, the functionality of these two complementary chimeras (HHMM and MMHH) for HIV-1 fusion (Fig. 3 and 4) and entry (Table 1; see below) indicates that none of the intracellular and transmembrane domains of mCCR-5 are detrimental for coreceptor activity when they are placed in a functional background. This analysis shows that a combination of any single extracellular domain of hCCR-5 placed in the nonfunctional mCCR-5 background is able to mediate HIV-1_{ADA} Env-induced fusion.

We then determined whether any domain of hCCR-5 was indispensable for the fusion process to occur. For that purpose, we sequentially replaced segments containing each extracellular domain of hCCR-5 by an mCCR-5 segment. The resulting chimeras, MHHH, HMHH, and HHMM (for the last, see above), behaved like hCCR-5 in ADA Env-induced fusion (Fig. 4), demonstrating that none of hCCR-5 sequences were essential for coreceptor function. The alternative explanation is that mCCR-5 sequences placed in a functional hCCR-5 background adopt a different conformation and can mediate some interaction with the fusion complex. We therefore conclude that multiple domains of hCCR-5 are involved in HIV-1_{ADA} Env-induced fusion. It was not possible to identify a single domain necessary and sufficient for fusion and likely to contain a binding site for the HIV-1 envelope.

Different HIV-1 isolates have distinct requirements for utilization of the CCR-5 coreceptor. Since different HIV-1 isolates can use the same coreceptor in distinct ways (66, 71), we extended our analysis to different primary SI and NSI isolates

TABLE 1. Infectivity of and syncytium formation by primary and TCLA HIV-1 isolates in cells transfected with different CCR-5 derivatives[a]

Viral isolate	No. of blue-stained syncytia per well[b] with transfected coreceptor:									
	None[c]	CXCR-4	hCCR-5	mCCR-5	HMMM	MHMM	HHMM	MHHH	HMHH	MMHH
NSI M-tropic[d]										
ADA	1	6	965	0	146	62	643	985	1,104	976
SF-162	0	0	848	0	161	302	420	904	991	867
M23	0	0	118	0	0	8	25	124	101	4
E80	0	0	92	0	0	3	30	128	123	0
SI dual-tropic										
89.6	0	662	158	0	5	1	1	6	21	2
GUN-1[e]	0	426	122	1	0	0	14	0	122	1

[a] Parental and chimeric receptors were transfected into CCC-CD4 cells, infected, and immunostained 3 days postinfection as described in Materials and Methods. Results of one of three independent experiments giving essentially the same results are presented.
[b] In 48-well plates.
[c] Transfection was with the empty pcDNA3 vector.
[d] SI status and tropism were previously determined (78).
[e] TCLA strain.

that use CCR-5 as a coreceptor and tested them in cell-free infection.

The panel of primary isolates used here have been previously described for their tropism, SI status, and coreceptor usage (78). The different hCCR-5–mCCR-5 chimeras were transiently transfected into nonpermissive feline CCC-CD4 cells before being challenged with cell-free viral supernatant. The cells were immunostained for intracellular p24 production 3 days postinfection. The results are summarized in Table 1. The ADA and SF-162 isolates were able to infect CCC-CD4 cells expressing each of the hCCR-5–mCCR-5 chimeric receptors tested, although at lower levels for HMMM and MHMM. These results parallel those obtained in the syncytium formation assay. Two other primary NSI M-tropic strains (M23 and E80) able to use the MHHH, HHMM, and HMHH chimeras were, however, unable to use HMMM, MHMM, and MMHH for efficient entry into transiently transfected CCC-CD4 cells.

Surprisingly, infection by the SI dual-tropic strains 89.6 and GUN-1 was more severely affected by modification of hCCR-5. Infection by 89.6 was efficient only in cells expressing hCCR-5 and CXCR-4, while GUN-1 was able to use only the HMHH chimeric receptor. These results were unexpected, since these isolates are able to interact with at least two divergently related chemokine receptors (CXCR-4 and CCR-5). The reason for this dependence on CCR-5 structure and sequence integrity is not yet clear but may relate to a particular envelope conformation and/or sequence of these dual-tropic isolates. The TCLA strain LAI is able to use only CXCR-4 as a coreceptor and did not use any of the chimeric CCR-5 receptors (data not shown). Since the ADA and SF-162 envelope glycoproteins were able to use all of the chimeras transfected into CCC-CD4, we assume that each receptor is expressed, folded, and transported correctly in this cell type as well and that the results observed are not cell type specific. However, variation in levels of surface expression of chimeras (particularly for HMMM) may account for some of the lower efficiency of entry observed for some isolates.

Our results show that coreceptor utilization is probably conformationally complex, involving multiple extracellular domains of CCR-5. Furthermore, different isolates are more sensitive than others to change in the sequence and/or conformation of the coreceptor. It is noteworthy that these requirements correlate with tropism, with SI dual-tropic isolates being more dependent on hCCR-5 integrity than NSI M-tropic isolates.

DISCUSSION

In this study, we were interested in trying to determine the extracellular domains of CCR-5 important for HIV-1 entry and fusion. For this purpose we used the murine homolog of CCR-5 that has recently been cloned (10, 57) and shown to be nonfunctional as a coreceptor for HIV-1 (6, 8). This molecule has 83% identity at the amino acid level with its human counterparts (Fig. 1) but is still not able to be used as a coreceptor by a range of primary M-tropic and dual-tropic HIV-1 isolates (Fig. 3 and 4 and Table 1). Despite their high level of similarity (91%), hCCR-5 and mCCR-5 have different activities in HIV-1 coreceptor function. A rhesus macaque homolog of CCR-5, which differs from hCCR-5 at eight amino acid positions, is able to function as an HIV-1 coreceptor (16, 54). Thus, the coreceptor activities of these nonhuman CCR-5 homologs in vitro correlates with their activities in vivo: $CD4^+$ mouse lymphocytes and CD4 transgenic mice are not infected by HIV-1, whereas macaque PBMCs and macrophages are infected in vitro and in vivo by a simian-human immunodeficiency virus recombinant that expresses the HIV-$1_{SF\text{-}162}$ M-tropic envelope glycoproteins (52).

Some of the changes in mCCR-5 are located in extracellular domains, suggesting that these differences may influence its function as an HIV-1 coreceptor. Subtle changes can have a dramatic effect on ligand interaction as exemplified by the chemokine binding profile of mCCR-5, which is unable to bind efficiently human MIP-1α but binds human MIP-1β and human RANTES at levels of affinity similar to those of the corresponding murine chemokines (57). Using six hCCR-5 and mCCR-5 chimeric receptors and testing them for HIV-1_{ADA} Env-induced cell fusion and cell-free infection with six different isolates, we showed that (i) several if not all extracellular regions of CCR-5 are implicated in the interaction with HIV-1 and (ii) there are different CCR-5 requirements for different isolates.

Using the ADA envelope glycoproteins in a syncytium formation assay, we showed that any combination of hCCR-5 segments placed in nonfunctional mCCR-5 was able to restore coreceptor function. Partial fusion was observed when we introduced a fragment spanning the human amino-terminal or E1 domain into mCCR-5. Unfortunately, reduced surface expression of HMMM may explain inefficient fusion and entry for this construct. Replacing fragments containing each extracellular domain of hCCR-5 with fragments containing the corresponding murine domain showed that none of the human domains was indispensable for the function. No single extracellular domain proved to be necessary or sufficient for CCR-5 to be used as an HIV-1 coreceptor. It was, however, not possible by this strategy to test the role of the E3 region, since this loop is identical between hCCR-5 and mCCR-5. Moreover, no important roles were noted for transmembrane and intracellular regions in HIV-1 coreceptor activity with the chimeras used here. The implication from these data is that the interaction between Env and CCR-5 involves more than one region of the coreceptor. Clearly, mCCR-5 is incapable of making some key interactions resulting in lack of fusion activity, but this deficiency can be repaired in several ways.

Using a panel of primary NSI M-tropic and SI dual-tropic isolates, we found that the requirements were not the same between these phenotypically different strains. Cell-free virus infection by the ADA and SF-162 isolates gave similar results in the syncytium formation assay. However, two other primary NSI M-tropic strains (M23 and E80) were able to fuse only with some of the chimeric coreceptors. For these two isolates, we were unable to challenge transfected cells with as high an input of virus as that used for ADA and SF-162. Thus, the lower levels of or lack of infection by these two viruses noted for some of the constructs may partly be due to the low viral input. In the case of MMHH, however, it is clear that while full infectivity by ADA and SF-162 was observed, M23 and E80 plating was at background levels. Further studies will be needed to assess the extent of variation in the use of CCR-5 between distinct NSI M-tropic strains. Bieniasz et al. recently showed a differential usage of CCR-5 between the two NSI M-tropic isolates ADA and Ba-L in cell-cell fusion that correlated to the V3 loop sequence (8).

In contrast, the SI dual-tropic strains generally could use few of the chimeric receptors. The strain specificity reported here was not totally unexpected, as it has been shown that different HIV-1 strains have different requirements for interaction with the N-terminal extracellular domain of CXCR-4 (66) or CCR-5 (71). A recent study using CCR-5–CCR-2b chimeras also showed that HIV-1 must interact with multiple domains, the N-terminal and E1 domains being the most important (71). Using the 89.6 dual-tropic isolates, Rucker et al. showed that

this isolate has requirements different from those of JR-FL for CCR-5 use. However, the interpretation of the results was complicated by the fact that the partner used for the chimeras was CCR-2b, which is also used as a coreceptor by 89.6. We showed here that the dual-tropic 89.6 and GUN-1 isolates were inefficient in using most of the hCCR-5–mCCR-5 chimeras for entry, despite the fact that the level of infection of CCR-5-expressing cells was the same as for M-tropic isolates.

The differences observed between NSI M-tropic and SI dual-tropic isolates for CCR-5 usage are surprising, since the latter isolates can use multiple coreceptors to gain entry into cells: 89.6 and GUN-1 can use CCR-5 and CXCR-4 (33, 78), and in addition, 89.6 can use CCR-3 and CCR-2b (34). Thus, they are able to use different divergent members of the chemokine receptor family for entry into cells and yet subtle changes in CCR-5 abolish their entry. In contrast, M-tropic isolates proved less sensitive to changes in CCR-5 despite the fact that their coreceptor usage is more restricted. This apparent paradox suggests that multiple ways of interacting with CCR-5 exist among HIV-1 strains, which may influence their ability to gain the use of other coreceptors. It is possible that evolution of a CCR-5-specific HIV-1 envelope to exploit more than one coreceptor may lower its affinity for CCR-5, making it more sensitive to changes in the sequence. The changes that take place in NSI isolate envelope glycoprotein sequences in vivo during the NSI-to-SI phenotypic switch (14, 18, 39, 49) must reflect the ability of their envelope glycoproteins to interact with CXCR-4 (46). These changes may not be compatible with interaction with CCR-5 at the same sites or with the same affinity. Alternatively, the ability of gp120 to interact with the coreceptor is necessary but not sufficient for the entry process to be completed.

It has recently been shown that sequences inside the V3 loop known to influence tropism (17, 21, 44, 56, 61, 75, 80, 85, 87) and post-CD4 binding events (7, 40, 45, 64) also influence coreceptor usage (22, 33, 67), formation of the CD4–CCR-5–gp120 ternary complex (81, 88), and sensitivity to β-chemokine-mediated inhibition of entry (27, 46). The V3 loop is therefore a candidate region for interaction with CCR-5 but is probably not the only Env region involved. In fact, it has been shown that a recombinant NSI M-tropic isolate containing a TCLA strain V3 loop was still able to infect PBMCs and macrophages. This recombinant probably still interacted with CCR-5 despite the TCLA strain-derived V3 loop sequence (15, 62). Other variable loops like V1 and V2 that influence tropism and the SI ability of HIV-1 (11, 47, 79) may also be involved. Other factors, such as a possible CD4–CCR-5 interaction, must also be taken into account to explain the precise mechanism of interaction of the HIV-1 envelope glycoproteins with the coreceptor. Results from a study by Wu et al. (88) showing that a soluble form of CD4 consisting of the two amino-terminal domains (D1D2 sCD4) can inhibit MIP-1α and MIP-1β binding to CCR-5 in the absence of gp120 support this possibility. Furthermore, differences in affinities for the binding receptor CD4 may also influence the way different HIV-1 strains interact with the receptor complex (48).

During the course of this work, two studies also reported by a similar approach that several CCR-5 extracellular domains were involved in HIV-1 entry (6, 8). In contrast to our results, Atchinson et al. (6) showed that an MMHH construct was only minimally active for Ba-L, while our equivalent construct fully supported entry of two NSI M-tropic isolates: ADA and SF-162. Bieniasz et al. using an envelope-mediated cell fusion assay reached conclusions similar to those of our study: they observed differential usage of hCCR-5 and mCCR-5 chimeras between distinct NSI M-tropic isolates (ADA and Ba-L) and restrictive utilization of the chimeric receptors by the 89.6 isolate. Our analysis further shows that this is also true in cell-free virus infection and that it is likely to be a general feature of dual-tropic strains to interact less efficiently or differently with CCR-5.

In summary, we have shown that multiple domains of CCR-5 are involved in HIV-1 entry and syncytium formation. Six different CCR-5-using isolates were tested and shown to vary in coreceptor requirements for cell entry. These distinct coreceptor needs correlated with tropism, with dual-tropic strains being more sensitive to changes in their CCR-5 sequences. It will be interesting to see how these dual-tropic isolates interact with the other major coreceptor, CXCR-4. Other chimeras and mutants of CCR-5 and CXCR-4 need to be tested to precisely understand the HIV-1 entry mechanism.

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