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Envelope Glycoproteins from Human Immunodeficiency Virus Types 1 and 2 and Simian Immunodeficiency Virus Can Use Human CCR5 as a Coreceptor for Viral Entry and Make Direct CD4-Dependent Interactions with This Chemokine Receptor

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Several members of the chemokine receptor family have recently been identified as coreceptors, with CD4, for entry of human immunodeficiency virus type 1 (HIV-1) into target cells. In this report, we show that the envelope glycoproteins of several strains of HIV-2 and simian immunodeficiency virus (SIV) employ the same chemokine receptors for infection. Envelope glycoproteins from HIV-2 use CCR5 or CXCR4, while those from several strains of SIV use CCR5. Our data indicate also that some viral envelopes can use more than one coreceptor for entry and suggest that some of these coreceptors remain to be identified. To further understand how different envelope molecules use CCR5 as an entry cofactor, we show that soluble purified envelope glycoproteins (SU component) from CCR5-tropic HIV-1, HIV-2, and SIV can compete for binding of iodinated chemokine to CCR5. The competition is dependent on binding of the SU glycoprotein to cell surface CD4 and implies a direct interaction between envelope glycoproteins and CCR5. This interaction is specific since it is not observed with SU glycoprotein from a CXCR4-tropic virus or with a chemokine receptor that is not competent for viral entry (CCR1). For HIV-1, the interaction can be inhibited by antibodies specific for the V3 loop of SU. Soluble CD4 was found to potentiate binding of the HIV-2 ST and SIV_{mac239} envelope glycoproteins to CCR5, suggesting that a CD4-induced conformational change in SU is required for subsequent binding to CCR5. These data suggest a common fundamental mechanism by which structurally diverse HIV-1, HIV-2, and SIV envelope glycoproteins interact with CD4 and CCR5 to mediate viral entry.

Infection with human immunodeficiency virus type 1 (HIV-1), HIV-2, and simian immunodeficiency virus (SIV) requires an interaction between the envelope glycoprotein surface (SU) component of the virus and CD4 on the plasma membrane of a target cell (79). Early studies suggested that additional target cell cofactors are also required to act in concert with CD4 to permit envelope-mediated fusion of viral and cellular membranes (3, 15, 44, 58, 60, 73). Recently, several members of the family of chemokine receptors were identified as the cofactors involved in HIV-1 entry. In general, HIV-1 strains phenotypically characterized as T-cell line tropic (T tropic) were shown to use the CXC chemokine receptor 4 (CXCR4) (31) for infection, and viruses phenotypically characterized as macrophage tropic (M tropic) were shown to use the CC chemokine receptor 5 (CCR5) for infection (1, 14, 25–27).

Chemokine receptors are seven-transmembrane G-protein-coupled receptors that, upon binding of their low-molecular-weight protein ligands, transmit signals that result in chemotactic responses of leukocytes (66, 67, 80). The CC chemokines MIP-1α, MIP-1β, and RANTES are ligands for CCR5 (19, 20, 76) and are able to inhibit infection by strains of HIV-1 that use CCR5 for viral entry (17). The CXC chemokine SDF1 is a ligand for CXCR4 and is able to inhibit infection by strains of virus that use this chemokine receptor for entry into cells (8, 69). Although the majority of HIV-1 strains analyzed to date use either one of these two receptors for entry, dual-tropic strains that use both receptors as well as strains that additionally use CCR3, or CCR3 and CCR2b, have also been identified (14, 26).

The cellular tropism of HIV-1 is determined by the structure of the envelope glycoprotein (Env) (11, 12, 56, 70), particularly by specific sequences within the V3 loop of the gp120 SU component (10, 13, 40, 45, 81, 89). These same structures also determine the viral specificity for CXCR4 or CCR5 (14).

HIV-2 and SIV, like HIV-1, are members of the primate lentivirus family and are more closely related to each other than to HIV-1 (41). Target cell tropism of HIV-2 and SIV is similarly dictated by the structure of the envelope glycoprotein (48). The Envs of HIV-2 and SIV show greater than 60% sequence similarity to each other but less than 40% similarity to HIV-1 Envs (41). Despite the differences between the HIV-1 Envs and the Envs from HIV-2 and SIV, the molecules are clearly related both structurally and functionally. The sequence homology is sufficient to suggest similar domain structures (43), and polyclonal sera to HIV-1 Env cross-react with HIV-2 and SIV Envs (35, 86). In addition, with some rare exceptions, all three virus families can use human CD4 for entry into cells, and the SU components of the Envs bind to CD4 (42, 77). Strains of HIV-2 and SIV infect a subset of human CD4-expressing cells that overlap but are distinct from those infected by HIV-1, suggesting the existence of specific entry cofactors for HIV-2 and SIV (15, 44).

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A role for CD4 in binding to HIV and SIV Env during viral infection has been established (58, 59, 77, 78), but the subsequent mechanisms by which CD4 and coreceptors mediate viral entry are still not understood. Recent data indicate that purified HIV-1 gp120 from virus strains that use CXCR4 can form a trimolecular complex with CD4 and CXCR4 (54). Other recent studies have shown that HIV-1 gp120s from CCR5-tropic strains can displace labeled β chemokines from CCR5 on $CD4^+$ cells (84). These Env glycoproteins interact poorly, if at all, with CCR5 alone but have a significant increase in affinity for the chemokine receptor in the presence of soluble CD4 (90). Additional data indicate that the membrane-distal D1 and D2 domains of CD4 may interact directly with CCR5 (90). Thus, at least part of the mechanism of viral entry after CD4 binding may involve CD4-induced direct interactions between gp120, chemokine receptors, and CD4.

The coreceptors for HIV-2 and SIV have not yet been extensively studied, but a role similar to that of chemokine receptors in HIV-1 infection can be inferred. A number of in vitro-selected strains of HIV-2 are able to infect cells which lack expression of CD4 (16, 28), and recently one of these strains was shown to use CXCR4 for CD4-independent viral entry (28). In addition, a number of strains of HIV-2 can infect cells lacking CD4 expression, but only in the presence of soluble CD4 (16). Soluble CD4 can also enhance infection of CD4-expressing cells by some strains of HIV-1, HIV-2, and SIV (2, 16, 83, 87). These combined data suggest that HIV-2 Env can adopt a conformation that allows direct interactions with a chemokine receptor and that CD4 can facilitate interactions of HIV-2 and SIV Envs with appropriate coreceptors during the process of viral entry into cells.

To further understand the role of CD4 and chemokine receptors in HIV-2 and SIV infection, we have used virus pseudotyped with Env from several strains of HIV-2 and SIV to screen a panel of cell lines expressing CD4 and individual human chemokine receptors. We have found that both HIV-2 and SIV Envs use CCR5 for infection, and some HIV-2 Envs use CXCR4. In addition, our data indicate that some strains of virus employ more than one coreceptor, some of which remain to be characterized. We have used purified SU glycoprotein from selected strains of CCR5-tropic HIV-1, HIV-2, and SIV to demonstrate that each of these makes direct CD4-dependent interactions with CCR5. For the HIV-2 and SIV Envs, this interaction was shown to be due to a CD4-induced increase in affinity for CCR5.

MATERIALS AND METHODS

Env expression plasmids. Plasmids encoding envelope glycoproteins from HIV-1 strains HXB2, JRFL, and BaL (45, 52, 88) have previously been described. HIV-2 clones ST (49), ST24.1C#2 (50), and JK7312A (33, 34) were the kind gifts of Beatrice Hahn and were used to derive Env expression plasmids ST24.1 and pJK7312A, respectively. The *env* genes were generated by PCR using the proviral DNA and were subcloned into the expression plasmid pSP272, obtained from Michael Emerman. The SIV_{mac239} and $SIV_{mac1A11}$ envelope expression plasmids have previously been described (91), and the $SIV_{agmTy01}$ expression plasmid was made by K. Zingler, using the pSM vector with proviral DNA supplied by Tomoyuki Miura. Expression plasmids for Envs from HIV-2 UC1 (29) and UC2 (7, 30) were a gift from Rika Furuta and Carol Weiss (Food and Drug Administration).

Chemokine receptor plasmids. Plasmids expressing wild-type chemokine receptors have previously been described (25). Chemokine receptors with green fluorescent protein (GFP) (23) fused to the carboxy terminus (CCR1 and CCR5) were made by the overlap PCR method. The last amino acid before the stop codon of each chemokine receptor was fused to a linker sequence GSGGTGSGP which was fused directly to the initiator methionine of GFP. CCR5 with the hemagglutinin (HA) epitope tag inserted 14 amino acids from the amino terminus (HA-CCR5) was a gift from Sunny Choe and Ned Landau (55).

Purified envelope SU proteins and sCD4. Purified HIV-1 gp120 from the BH10, JRFL, and BaL strains and HIV-2 gp120 from the ST strain were prepared from transfected *Drosophila melanogaster* Schneider 2 cells (9, 46) by J. Culp and B. Hellmig and were the kind gift of Ray Sweet, SmithKline and Beecham. Purified soluble CD4 (sCD4) (24) was also provided by Ray Sweet. The SIV_{mac239} gp130 was purified from Chinese hamster ovary cells by Bio-Molecular Technology Inc. and was obtained from the Division of AIDS, National Institute of Allergy and Infectious Diseases, through the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases.

Antibodies. Leu3a and Leu2 were purchased from Becton Dickinson and were dialyzed against azide-free phosphate-buffered saline (Gibco) with 0.1% bovine serum albumin (BSA; Sigma) before use. OKT4 was a gift from Procept Inc. (Cambridge, Mass.). The anti-gp120 antibodies have been described previously; 447 is 447-52-D (36, 39), 257 is 257-D (38, 39), 1027 is 1027-15D (37), and 670 is 670-D (92).

Cell culture and preparation of cell lines expressing chemokine receptors. All cell lines were grown in Dulbecco modified Eagle medium (Gibco) with 10% fetal calf serum (Atlanta Biologicals), supplemented with antibiotics.

U87-MG mega-glioblastoma astrocytic cells were obtained from the American Type Culture Collection. U87 cells expressing CD4 were made as previously described (51, 74). Briefly, 24 h prior to transfection, 3×10^6 cells of the murine leukemia virus amphotropic packaging cell line Bing (74) were plated on 10-cm-diameter dishes. The cells were transfected by a modification of the $CaPO_4$ method (51) with 20 μg of the defective retroviral vector pMV7, encoding a human CD4 cDNA insert (53). At 48 h after transfection, virus was harvested from the cells and used to infect 10^6 U87 cells in 10-cm-diameter dishes; 48 h later, cells were selected with Geneticin (Gibco BRL) at 300 μg/ml. After 10 days of growth in selective medium, the bulk population of Geneticin-resistant cells were stained with a phycoerythrin (PE)-conjugated anti-CD4 antibody (Leu-3a PE; Becton Dickinson) and were found to be 100% CD4 positive by fluorescence-activated cell scanner (FACScan; Becton Dickinson) analysis. U87 and U87.CD4 cell lines expressing individual chemokine receptors were established in a similar manner except that the cDNAs were cloned into the defective retroviral vector pBABE-puro (63) and U87 and U87.CD4 cells were selected with puromycin at 0.5 μg/ml. Expression of CCR5 and CXCR4 at the cell surface of both U87 and U87.CD4 cells was confirmed by using antibodies (provided by Charles McKay and Jim Hoxie) specific for these chemokine receptors (data not shown). Bulk populations of selected cells were then used in infection assays.

3T3.CD4 cells were generated by techniques similar to those used for U87 cells. The defective retroviral vector pMX (72) containing a CD4 cDNA insert was transfected into Bing cells, and the virus from these cells was used to infect 5×10^5 3T3 cells in a 10-cm-diameter dish. The pMX vector does not contain a gene for antibiotic selection of transfected cells; however, the multiplicity of infection obtained was sufficiently high such that after a week of growth, FACScan analysis revealed that the cells were 100% positive for CD4. 3T3 and 3T3.CD4 cells expressing chemokine receptors were generated by techniques similar to those used above. The chemokine receptors used in the 3T3 cells were CCR1, CCR5, or CXCR4, fused at the carboxy terminus to GFP, or HA-CCR5 (see above). The defective retroviral vector pBABE-puro containing cDNA inserts for each of the tagged chemokine receptors was transfected into Bing cells to make virus stocks which were used to infect 3T3 and 3T3.CD4 cells. At 48 h after infection, the cells were placed under selection in puromycin (3 μg/ml). A week later, GFP-expressing cells were analyzed directly on the fluorescein isothiocyanate channel of the FACScan, and HA-CCR5-expressing cells were incubated with an anti-HA antibody (Boehringer Mannheim), followed by a PE-conjugated anti-mouse antibody (Caltag), and analyzed on the FACScan. The cell populations were between 80 and 100% positive for the tagged chemokines. All cell lines were subsequently sorted on a Coulter fluorescence-activated cell sorter to obtain cells with up to threefold-higher levels of expression than the initial populations.

293T cells were transiently transfected by a modification of the $CaPO_4$ method (53). Briefly, 24 h prior to transfection, 3×10^6 cells were plated on 10-cm-diameter dishes. Cells were cotransfected with 10 μg of vector pCD T4 (53) and 10 μg of individual pBABE-puro plasmids containing inserts for the HA- and GFP-tagged forms of the chemokine receptors. The GFP-tagged chemokine receptors have the same entry cofactor specificity for viral Envs as the wild-type chemokine receptors (data not shown). Cells were harvested 24 h after transfection and replated for subsequent infection with pseudotyped virus at 48 h after transfection.

Pseudotyped virus infection assays. NL4-3-Luc-R^-E^- (21) virus stocks pseudotyped by different Envs were generated by transfecting 293T cells with 10 μg of pNL4-3-Luc-R^-E^- and 10 μg of envelope expression plasmid. Virus-containing supernatants were harvested 48 h later, clarified of contaminating cell debris, and frozen at −80°C. Mixed viral particles of HIV-2 ST and NL4-3-Luc-R^-E^- were made by transfection into 293T cells of 10 μg of each proviral DNA and harvesting of the supernatants as described above. Viruses were quantified by p24 enzyme-linked immunosorbent assay (Cellular Products Inc.). Target cells were plated in 24-well plates at 10^5 per well for U87 or at 5×10^4 per well for 293T. Cells were infected 24 h after plating with 0.5 ml of luciferase reporter virus per well; 20 ng of p24 per well was used for Env-pseudotyped virus, and 100 ng of p24 per well was used for HIV-2 ST–NL4-3-Luc-R^-E^- mixed viral particles. After 16 h, 1 ml of fresh medium was added to each well; 48 to 60 h later, the cells were harvested and assayed for luciferase activity, using a Promega luciferase assay kit and a Wallac Microbeta 1450 Counter.

Chemokine binding and displacement assays. ^{125}I-MIP-1α and ^{125}I-MIP-1β (2,000 Ci/mmol and 25 μCi/ml) were purchased from Dupont NEN. Binding assays were performed by using a modification of the method of Meyer et al. (61). Briefly, assays were performed in round-bottom 96-well plates in a volume of 40 μl of assay buffer. Assays were performed in one of two different buffers. Experiments with cells expressing cell surface CD4 were performed with Hanks buffered saline solution (Gibco BRL) containing 5 mg of BSA per ml. Experiments using sCD4 were performed with a HEPES-based buffer which consisted of 50 mM HEPES (pH 7.0), 5 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mg of BSA per ml. 3T3 cells were removed from tissue culture dishes with 2 mM EDTA in phosphate-buffered saline and then plated at 2×10^5 cells per well. Purified envelope SU proteins were added to cells in a final volume of 20 μl and at various concentrations up to 400 nM. The wells of the plate were sealed with a plate sealer and incubated for 1 h at room temperature prior to the addition of 20 μl of iodinated chemokine at 0.25 to 0.1 nM, depending on the experiment. The plate was resealed and transferred to 37°C for 1 h. Cells were then transfered to a Millipore 96-well filtration plate (MADV NOB10) and vacuum applied by using a Millipore Multiscreen assay system vacuum manifold. The cells were washed twice with 200 μl of assay buffer supplemented with 0.5 M NaCl. The plates were dried, and 40 μl of scintillation fluid was added to each well, and the plates were counted in a Wallac Microbeta 1450 Counter.

RESULTS

Chemokine receptor specificities of HIV-2 and SIV envelopes. Envs from a variety of HIV-1, HIV-2, and SIV strains were used to produce pseudotyped virus with an Env-defective HIV-1 NL4-3 provirus engineered to express a luciferase reporter gene (21). Incubation of cells with these pseudotyped virions results in a single round of infection and subsequent expression of the luciferase gene after viral integration. Determination of luciferase activity in infected cells provides a highly sensitive, quantitative measure of viral entry (21).

Initially we tested each of the pseudotyped viruses on a panel of U87 cell lines expressing human CD4 and individual chemokine receptors (Fig. 1). Consistent with previous results, virus pseudotyped with Envs from M-tropic HIV-1 strains JRFL and BaL infected cells expressing CCR5, while virus bearing Env from the T-tropic HXB2 strain infected CXCR4-positive cells (Fig. 1A).

The HIV-2 Envs used to generate pseudotyped virus were from genetically diverse strains of HIV-2 subgroup A (ST24.1, UC2, and ROD), subgroup B (UC1), and a recombinant virus from subgroups A and B (p7312A) (33, 34). In addition, the HIV-2 strains appear to differ in their cellular tropism, but this has been characterized to different extents for the various strains. HIV-2 JK7312A exhibits a tropism for activated peripheral blood mononuclear cells (PBMC) but not for transformed T-cell lines or macrophages (47a). In contrast, HIV-2 ST24.1, ROD, and UC2 can be grown on activated PBMC and transformed T-cell lines such as SupT1 and Hut 78 (7, 27a, 30, 49). Finally, UC1 productively infects macrophages as well as transformed T-cell lines and PBMC (29).

Interestingly, despite the genetic and phenotypic differences between HIV-2 JK7312A, ST-24.1, and UC1, Envs from each of these strains used CCR5 for entry into CD4-positive cells (Fig. 1B). UC2 Env was found to use CXCR4, while HIV-2 ROD Env displayed tropism for multiple receptors, including CCR3, CXCR4, and, weakly, CCR5.

In subsequent experiments (see below), we wished to examine potential interactions between CCR5 and purified SU glycoprotein from HIV-2 ST. This virus is the parental strain of HIV-2 ST24.1 and has the same tropism but grows more slowly and does not induce cytopathic effects in host cells such as SupT1 (50). To determine the chemokine receptor tropism of HIV-2 ST, we generated mixed viral particles containing HIV-2 ST and NL4-3-Luc-R^-E^- genomes (Materials and Methods). Infection with these particles of the panel of U87 cells expressing individual chemokine receptors indicated that the ST Env, as well as ST24.1 Env, was able to use CCR5 for viral entry (Fig. 1C).

The SIV Envs that we tested were also from diverse strains of virus. SIV_{mac239} and $SIV_{mac1All}$ are in the SIV_{mac}/SIV_{sm}/HIV-2 subgroup of viruses distinct from the SIV_{agm} subgroup (41). Each of the SIV strains shows tropism for human transformed T-cell lines (e.g., Hut 78) and host PBMC (32, 57, 68, 71, 85, 91), but in addition, $SIV_{mac1A11}$ can productively infect rhesus macaque macrophages (5, 6, 85). Interestingly, the Env from SIV_{agm} was clearly able to mediate infection of U87.CD4 cells in the absence of any exogenous chemokine receptor (Fig. 1D). However, CD4 expression alone was not sufficient to allow infection of 3T3 or 293T cells by SIV_{agm} Env-pseudotyped virus (data not shown). In infection of U87.CD4 cells SIV_{mac239} and $SIV_{mac1A11}$ used specifically CCR5 for infection, as did SIV_{agm}, although this was harder to distinguish against the background infection of U87 cells alone (Fig. 1D).

Displacement by Env of MIP-1α from CCR5-expressing cells. To determine whether the SU protein of viruses that utilize CCR5 for entry makes direct contact with the chemokine receptor, we assayed the ability of SU to compete for binding of iodinated MIP-1α to 3T3 cells expressing human CD4 and HA-CCR5 (55). The CCR5-specific SU proteins (gp120 of HIV-1 and HIV-2 and gp130 of SIV) used were from HIV-1 JRFL and BaL, HIV-2 ST, and SIV_{mac239}. As a control, we used gp120 from the CXCR4-tropic HXB2 virus (BH10). The HA-CCR5 was expressed at a higher level on 3T3 cells compared to wild-type CCR5 and hence was more convenient for use in the displacement assays (data not shown). For a negative control, we used 3T3.CD4 cells expressing human CCR1-GFP, which also binds MIP-1α but does not function as a cofactor for HIV or SIV entry (Fig. 2 and data not shown).

To confirm the function of HA-CCR5 as a coreceptor for infection (55) with the CCR5 tropic Envs that we used in our binding assays, we transiently transfected 293T cells with human CD4 and HA-CCR5. Infection by the luciferase reporter virus HIV-Luc pseudotyped with Envs from the three CCR5-tropic viruses HIV-1 JRFL, HIV-1 BaL, and SIV_{mac239} clearly indicated the use of HA-CCR5 as a coreceptor (Fig. 2A). As a control in these experiments, we used CCR5-GFP, which expresses the same extracellular domains as wild-type CCR5 and is equivalent in infection assays in 293T cells with each of these viruses (Materials and Methods and data not shown). As in Fig. 1C, in order to examine the use of HA-CCR5 by HIV-2 ST Env, we used mixed viral particles containing HIV-2 ST and NL4-3-Luc-R^-E^- genomes. As in Fig. 1, the signal obtained with these particles was approximately 10 times lower than that obtained with HIV-Luc pseudotyped by using Env expression plasmids; nevertheless, it is clear that HA-CCR5 and CCR5-GFP are used equivalently by HIV-2 ST Env, and the signal obtained was consistently 10-fold greater than the signal obtained in assays using CCR1-GFP.

Scatchard analysis of MIP-1α binding to 3T3.CD4 cells expressing each of the chemokine receptors showed approximate affinities of 0.3 nM for HA-CCR5 and 0.6 nM for CCR1-GFP. The numbers of binding sites per cell were 30,000 for HA-CCR5 and 200,000 for CCR1-GFP (data not shown). When purified SU protein from CCR5-tropic viruses was included in the binding assay, there was titratable displacement of ^{125}I-MIP-1α from 3T3.CD4 cells expressing HA-CCR5 (Fig. 3A). The gp120 from HIV-1 JRFL was the most efficient at MIP-1α displacement, while SUs from HIV-1 BaL, HIV-2 ST, and SIV_{mac239} were 20- to 80-fold less efficient. In contrast, incubation of purified gp120 from an HIV-1 T-tropic virus, BH10 (HXB2), had no effect on MIP-1α binding (Fig. 3A). In addition, none of the envelope glycoproteins was able to displace

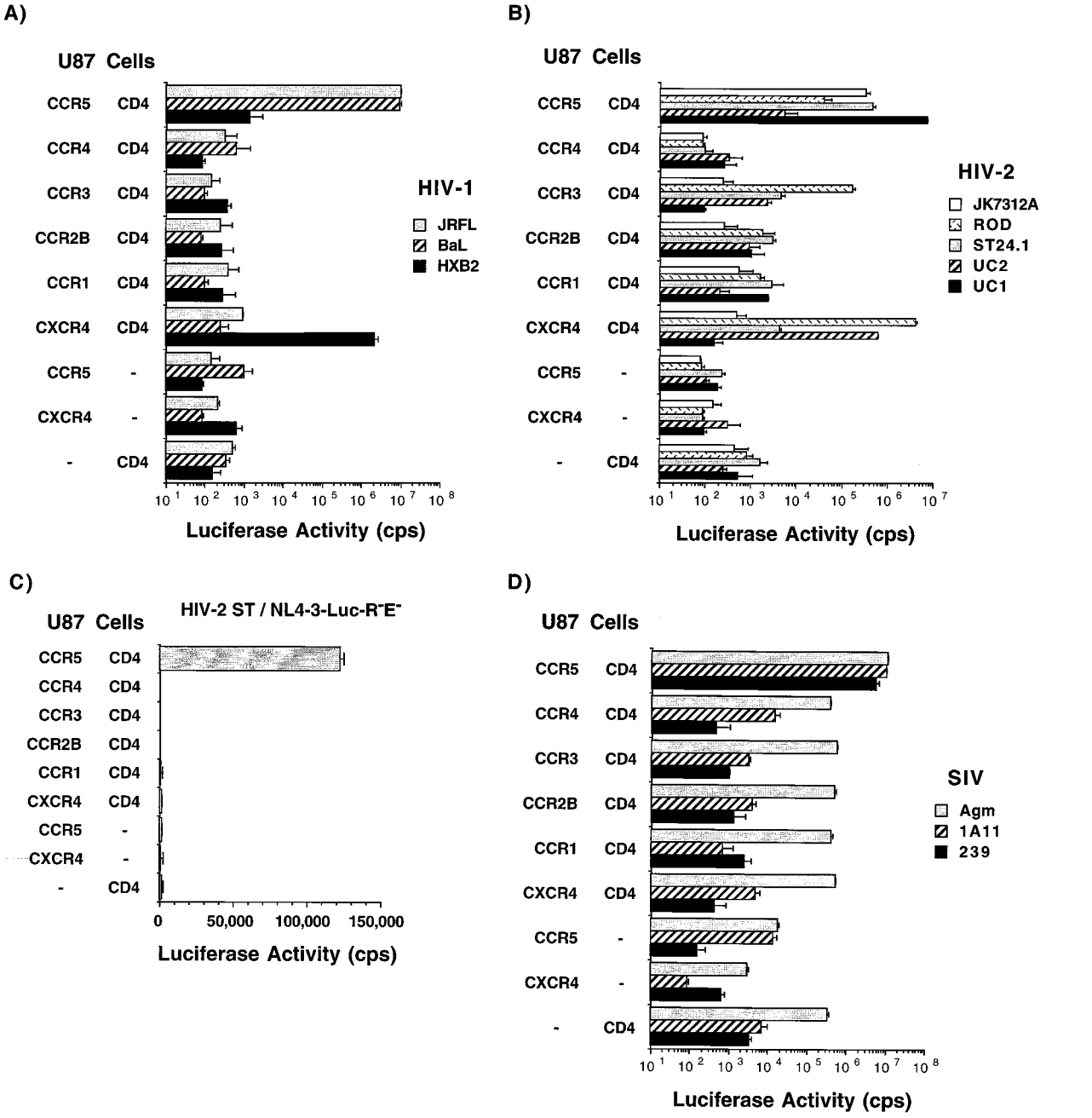

FIG. 1. Chemokine receptor utilization by envelope glycoproteins from diverse strains of HIV-1, HIV-2, and SIV. (A) Infection of U87 and U87.CD4 cells expressing individual chemokine receptors with NL4-3-Luc-R$^-$E$^-$ (HIV-Luc) pseudotyped with different HIV-1 Envs (20 ng of p24 per well). Cells were harvested 3 days after infection, and luciferase activity was determined and is expressed in arbitrary units (counts per second [cps]). (B) Infection with virus pseudotyped with HIV-2 Envs. (C) Infection with mixed viral particles prepared by transfection of HIV-2 ST and NL4-3-Luc-R$^-$E$^-$ proviral DNAs into cells (100 ng of p24 per well) (see Materials and Methods). (D) Infection with virus pseudotyped with SIV Envs (20 ng of p24 per well).

MIP-1α from CCR1-GFP (Fig. 3B). Experiments in which iodinated MIP-1β was used instead of MIP-1α for binding to HA-CCR5 yielded similar results (data not shown).

Interaction of gp120/gp130 with CCR5 is dependent on binding to CD4. To determine whether the displacement of labeled chemokine depended on a specific interaction between cell surface CD4 and the envelope glycoprotein, anti-CD4 antibodies were included in the incubations. Addition of the anti-CD4 antibody Leu3a at saturating concentrations resulted in a recovery of iodinated MIP-1α binding in the presence of Env from CCR5-tropic viruses (Fig. 4). This antibody is known to inhibit the interaction between gp120-gp130 and CD4 and to be able to inhibit infection by HIV and SIV (77, 78). In contrast, addition of the antibody OKT4, which does not block gp120-gp130 interactions with CD4 (77, 78), or an isotype-matched control antibody, had no effect on the inhibition of MIP-1α binding by CCR5-tropic gp120-gp130 (Fig. 4).

Anti-V3 antibodies inhibit gp120-CCR5 interactions. Variation in the structure of the V3 loop of HIV-1 gp120 results in the use of different chemokine receptors as cofactors for HIV-1 infection (14). In addition, anti-V3 loop antibodies have been shown to inhibit infection with both T-tropic and M-

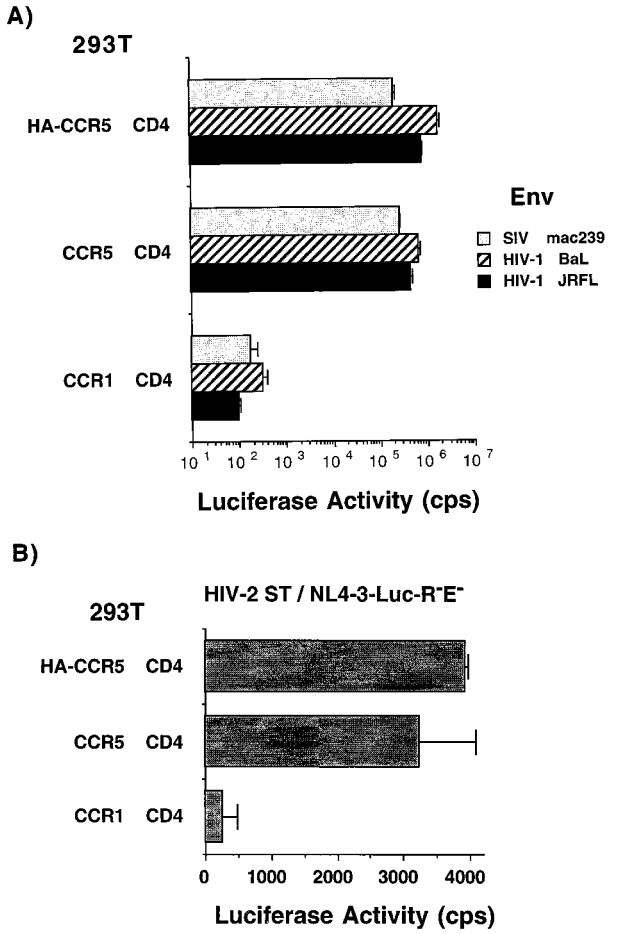

FIG. 2. Comparison of the abilities of HA-CCR5, CCR5-GFP, and CCR1-GFP to mediate infection with Envs from HIV-1, HIV-2, and SIV. (A) Infection of 293T cells, transiently transfected with chemokine receptor and CD4 expression plasmids, with HIV-Luc pseudotyped with the indicated envelope glycoproteins as in Fig. 1 (20 ng of p24 per well). (B) Infection with mixed viral particles as in Fig. 1C (100 ng of p24 per well).

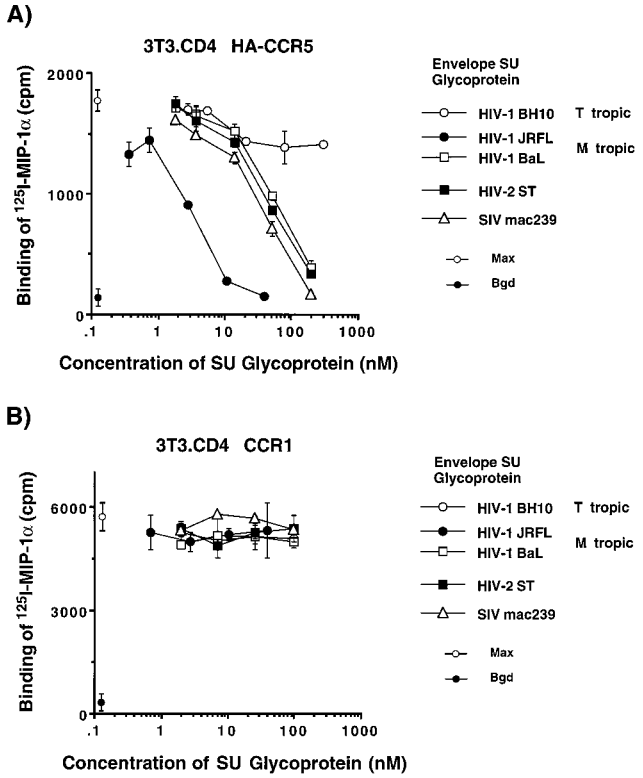

FIG. 3. Inhibition of MIP-1α binding to HA-CCR5 on 3T3.CD4 cells by SU glycoproteins from HIV-1, HIV-2, and SIV. 3T3.CD4 cells expressing (A) HA-CCR5 or (B) CCR1-GFP were incubated with increasing concentrations of SU glycoproteins as indicated, followed by incubation with 0.25 nM ^{125}I-MIP-1α, and processed as detailed in Materials and Methods. Bgd, counts in the presence of a 200-fold molar excess of cold MIP-1α; Max, counts bound in the absence of any additions other than binding buffer.

tropic HIV-1 (36, 62, 82). We therefore wished to determine if antibodies to the V3 loop of gp120 would affect the gp120-CCR5 interactions that we were measuring. Several antibodies specific for the V3 loop of HIV-1 gp120 were tested for the ability to bind to the JRFL viral particles (data not shown). Three that bound well (447, 257, and 1027) were added to the MIP-1α displacement assay with the HIV-1 gp120s from JRFL and BaL. Addition of each of the anti-V3 loop antibodies resulted in recovery of MIP-1α binding to HA-CCR5 (Fig. 5). In contrast, addition of an antibody specific for the C5 domain of gp120 (670) had no effect on MIP-1α displacement mediated by either JRFL or BaL.

Complexes of sCD4 and SU envelope glycoprotein have higher affinity for CCR5 than either component alone. The dependency of the Env SU-CCR5 interaction on binding of SU to cell surface CD4 may reflect at least two different functions of CD4. First, CD4 may be required to concentrate Env (and, by extension, virus particles) at the cell surface, thus increasing the probability of a productive interaction between Env and CCR5. Second, CD4 and gp120 could form a complex which has an increased affinity for CCR5 compared to gp120 alone, and this complex would then bind to CCR5. To determine whether a complex of CD4 and gp120 has increased affinity for CCR5, we measured iodinated MIP-1α binding to 3T3 cells expressing HA-CCR5 alone, in the presence of SU envelope glycoprotein with and without sCD4. We wished to use concentrations of CD4 that would be 10 times greater than the K_d of the SU-CD4 interaction and would also be at a molar concentration equivalent to or greater than the concentration of SU used. In this way, the concentration of CD4-SU complexes would be reflected by the concentration of SU added. Each of

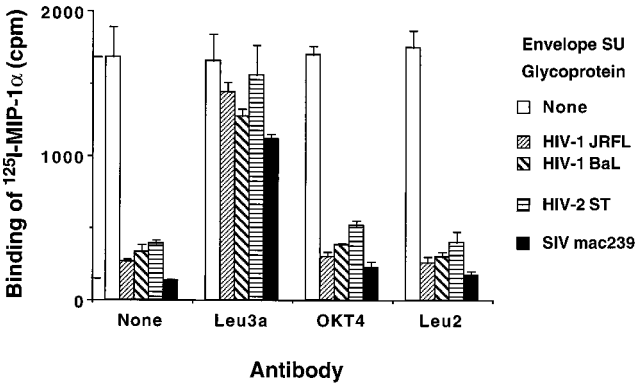

FIG. 4. Effects of anti-CD4 antibodies on the ability of SU to inhibit MIP-1α binding to HA-CCR5. 3T3.CD4 cells expressing HA-CCR5 were incubated at 4°C with anti-CD4 at saturating concentration or control antibodies at equivalent concentrations for 15 min prior to addition of 200 nM of SU glycoproteins and subsequent addition of 0.25 nM iodinated MIP-1α. The assay was processed as detailed in Materials and Methods.

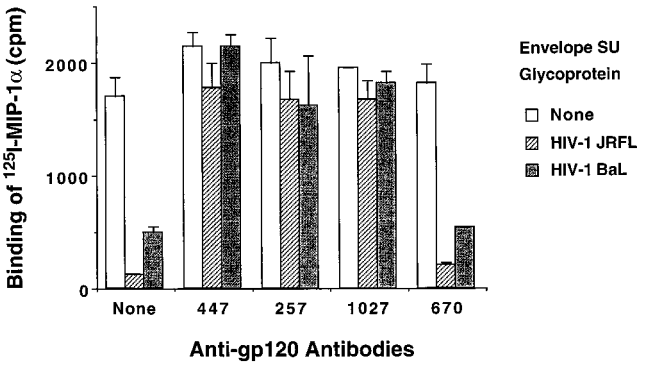

FIG. 5. Effects of anti-V3 loop antibodies on the ability of HIV-1 gp120 to inhibit MIP-1α binding to HA-CCR5. HIV-1 BaL (200 nM) and JRFL (100 nM) gp120s were incubated with the antibodies indicated at 15 μg/ml for 30 min at 4°C prior to addition to 3T3.CD4 cells expressing HA-CCR5 and subsequent addition of 0.25 nM iodinated MIP-1α. The assay was processed as detailed in Materials and Methods.

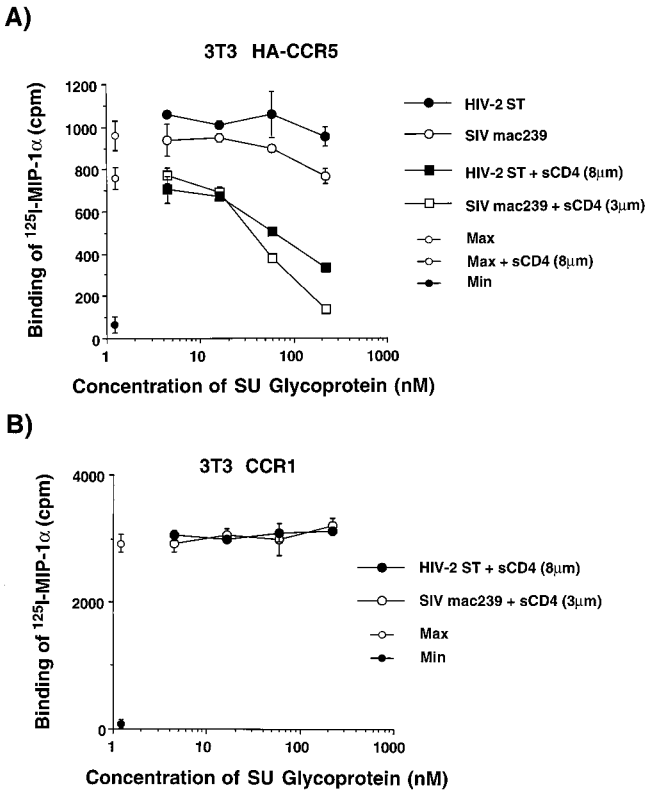

FIG. 6. Inhibition of MIP-1α binding to HA-CCR5 (A) or CCR1-GFP (B) by complexes of sCD4 and SU glycoproteins from HIV-2 ST and SIV_{mac239}. The indicated concentrations of SU were incubated with 8 μM (HIV-2) and 3 μM (SIV) of sCD4 at 4°C for 15 min prior to addition to 3T3 cells expressing HA-CCR5 for 1 h at room temperature followed by addition of 0.1 nM iodinated MIP-1α. The assay was processed as detailed in Materials and Methods.

the SU envelope glycoproteins was used at a maximum concentration of 200 nM. The K_ds of interaction between CD4 and the gp120 of HIV-1 JRFL and BaL are 0.2 and 30 nM, respectively (9, 46), and we therefore used 200 nM sCD4 in experiments with these gp120s. In contrast, the K_d of the CD4 interaction with SIV_{mac239} gp130 is 350 nM, and with HIV-2 ST it is greater than 1.4 μM (46). We therefore used 3 μM sCD4 for experiments with SIV_{mac239} gp130 and the maximal concentration of sCD4 that we could conveniently use (8 μM) in experiments with the ST Env.

In the presence of a constant amount of sCD4, we observed a titratable inhibition of chemokine binding with increasing concentrations of SU protein from SIV_{mac239} and HIV-2 ST (Fig. 6A). However, we have been unable to observe any MIP-1α displacement in the presence of 200 nM sCD4 and 200 nM HIV-1 gp120s (data not shown). The inhibition with HIV-2 and SIV SU appeared to be specific since there was no decrease in MIP-1α binding to CCR1-GFP-expressing cells in the presence of sCD4 and envelope glycoprotein (Fig. 6B). Occasionally, we have observed a slight decrease in MIP-1α binding in the presence of sCD4 alone, but this has not been consistently reproducible and has never been more than 20% of the maximal MIP-1α binding (Fig. 6A).

DISCUSSION

In this study, we have shown that envelope glycoproteins from diverse strains of HIV-1, HIV-2, and SIV are able to use CCR5 and that Envs from strains of HIV-1 and HIV-2 are able to use CXCR4 as cofactors for entry into CD4-positive target cells. Our results are consistent with previous studies on the cellular tropism of these viruses and also indicate that SIVs and, possibly HIV-2, can use other unidentified cofactors for infection of CD4-positive cells. In addition, we have demonstrated that SU components (gp120 and gp130) of several of the CCR5-tropic envelope glycoproteins interact directly with CCR5 on the cell surface, in a process that requires binding to CD4. These physical interactions are specific, since they are not observed with gp120 from Envs that utilize CXCR4 or with chemokine receptors that are not competent as cofactors for entry. For HIV-1 envelope glycoproteins, the interaction with CCR5 can be inhibited by antibodies specific for the V3 loop of gp120. Results obtained with the SIV_{mac239} and HIV-2 ST Envs further suggest that binding of these glycoproteins to CD4 results in the formation of a complex with increased affinity for CCR5.

Several of the diverse HIV-2 and SIV Envs that we examined were able to use specifically CCR5 as a cofactor for viral infection. Interestingly, a number of the CCR5-tropic Envs are from viruses that can be grown on transformed T-cell lines that are not infectable by CCR5-tropic HIV-1. For example HIV-2 ST, ST24.1, and UC1 can be grown on SupT1 cells (7, 30, 49), and SIV_{mac239}, SIV_{A11}, and SIV_{agm} can be grown on Hut 78 cells (32, 57, 68, 71, 85, 91). These viruses may therefore use an unidentified cofactor present on some human transformed T-cell lines. In addition, the Envs of several of these viruses, particularly that of SIV_{agm}, permit efficient entry into the glial cell line U87.CD4 in the absence of transfected coreceptors. The use of such additional coreceptors may allow a broad tissue tropism of the virus during infection, although the importance of each of the SIV and HIV-2 coreceptors in vivo remains to be determined. These data indicate that a single envelope glycoprotein can use more than one coreceptor, as demonstrated explicitly by the Env from HIV-2 ROD, which uses CCR5, CCR3, and CXCR4 for entry into cells. Similar multitropic viruses have been identified previously for HIV-1 and have been hypothesized to represent viruses that are transitional in their tropism between CCR5 and CXCR4 (18, 26). The existence of such viral Envs suggests that they have evolved to interact with structural components common to different chemokine receptors.

HIV-2 JK7312A is able to infect PBMC but not macrophages (47a), yet our data indicate that its Env uses CCR5,

which is expressed on both cell populations. These results suggest, therefore, that in addition to a requirement for CD4 and CCR5, there may be further restrictions to infection of macrophages by some HIV-2 strains. Similarly, SIV_{mac239} is unable to infect rhesus macaque macrophages (5, 6, 57, 65) although it can infect macaque PBMC. This restriction to macrophage infection by SIV_{mac239} has been shown to be determined by sequences in Env and to occur after viral entry (64). Further investigation will be required to determine whether a similar restriction maps to the Env of JK7312A and whether it occurs at the level of viral entry or at some later point in the viral life cycle.

The importance of Env binding to CD4 on the surface of target cells during viral infection has been well established (77), but the molecular mechanism by which chemokine receptors and CD4 act in concert to mediate viral entry has only begun to be defined. We have demonstrated that SU glycoproteins from CCR5-tropic Envs make CD4-dependent specific interactions with CCR5, suggesting that similar interactions occur during virus entry. Intriguingly, envelopes with a low affinity for CD4, such as those from SIV_{mac239} (K_d of 350 nM) and HIV-2 ST (K_d of >1.4 μM), are as efficient as Envs with a high affinity for CD4, such as HIV-1 BaL (K_d of 30 nM), in their interaction with CCR5 (Fig. 3). One possible explanation is that subsequent to CD4 binding, the Envs with lower affinity for CD4 have a higher affinity for CCR5. Consistent with this hypothesis, sCD4-SIV_{mac239} gp130 complexes were able to bind HA-CCR5 alone, whereas sCD4 complexes with gp120 of HIV-1 BaL could not (data not shown). Further quantitative studies will be required to test this model, but these data suggest that the effectiveness of Env-mediated interactions with target cells may be a function of the relative affinities for both CD4 and coreceptors on the surface of the cells.

Through its relatively high affinity for Env, CD4 is likely to have an important function in concentrating viral particles at the cell surface. As noted above, this function may be more significant for HIV-1 than for HIV-2 and SIV. Our experiments with complexes of soluble CD4 and purified SU from HIV-2 ST and SIV_{mac239} indicate that such complexes have a higher affinity for CCR5 than CD4 or SU alone. These data are consistent with previous observations that sCD4 can induce infection of $CD4^-$ cells with HIV-2 and can also enhance infection of $CD4^+$ cells with several strains of HIV-1, HIV-2, and SIV (2, 16, 83, 87). Recently Wu et al. demonstrated that complexing of sCD4 with gp120 of HIV-1 JRFL and BaL results in increased affinity for CCR5 (90). Using a similar assay system and similar conditions, we have been unable to demonstrate binding of these complexes to CCR5. This discrepancy is most likely due to differences in the cell lines used (murine lymphocytes versus murine 3T3 cells expressing CCR5) or in the form of epitope-tagged CCR5 used (N-terminal FLAG tag versus an internal HA tag). Alternatively, the assay system that we use may be less sensitive than that of Wu et al., and thus we detect only binding of sCD4-SU complexes of HIV-2 and SIV because they have a higher affinity for CCR5 than complexes of sCD4 with HIV-1 gp120. A thorough comparison of the relative affinities of these different complexes for CCR5 will be required to resolve this issue. Nevertheless, the combined data from our experiments and those of Wu et al. suggest that HIV-1, HIV-2, or SIV Env binding to CD4 leads to high-affinity interactions of CD4-Env with CCR5 and to subsequent membrane fusion.

The affinity of CD4-Env complexes for CCR5 may involve conformational changes in SU, as implied by changes in accessibility of epitopes within gp120 after it binds to sCD4 (47). Alternatively, there may be changes in the conformation of both CD4 and gp120, resulting in a novel surface for binding to the chemokine receptor. The recent demonstration that a soluble two-domain (D1D2) CD4 molecule, but not the full-length CD4, bound CCR5 directly suggests that upon gp120 binding, CD4 (D1D2) may adopt a specific conformation that can interact with CCR5 (90). It seems unlikely, however, that all binding to CCR5 would occur through CD4; the occurrence of viruses selected for CD4-independent entry suggests that Envs have a general propensity to interact with chemokine receptors (28). Further experiments will be required to determine the relative roles of interactions between CD4, Env, and CCR5 in viral entry and whether similar mechanisms are involved in Env interactions with other chemokine receptors.

The HIV-1, HIV-2, and SIV Envs that we have examined have less than 30% amino acid identity yet are able to use the same cofactor for viral entry. This finding suggests that there must be common structures within these diverse Envs which interact with CCR5. The ability of antibodies to the V3 loop of HIV-1 to inhibit interactions with CCR5 is consistent with the role of this region in determining the tropism of envelope glycoproteins for CCR5 versus CXCR4. However, the sequences of V3 loops from different M-tropic HIV-1 strains, as well as the sequences of the equivalent regions from HIV-2 and SIV, are divergent. This finding suggests that if this region makes a direct interaction with CCR5, there must be conserved structural elements in this region that are important for the interaction. More likely, this region may be part of a conformationally complex determinant which interacts with CCR5. Wu et al. have shown that antibodies to epitopes that are distinct from V3, and which are induced by sCD4, can also inhibit interactions between gp120-CD4 complexes and CCR5 (90). Establishing the molecular details of these interactions will require further mutagenesis and epitope mapping studies of gp120, CD4, and CCR5 itself. Recent mutagenesis studies of CCR5 in infection assays have indicated that interactions between the virus and CCR5 are complex and may involve multiple conformational determinants (4, 75). This may reflect more than the complexity of CD4-gp120 interactions with CCR5, since there must be subsequent changes in Env structure to allow interaction of the fusion peptide of gp41 with the cellular membrane. The highly hydrophobic environment of the chemokine receptors may create access to the cell membrane and hence may be involved in specific interactions with gp41.

The data that we have presented indicate a common mechanism by which HIV-1, HIV-2, and SIV use CD4 and CCR5 for entry into cells. In the simplest model, viral Env binding to CD4 results in a high-affinity complex for coreceptor in the cell membrane and subsequent conformational changes in Env lead to exposure of the gp41 fusion domain and to fusion of the viral and cellular membranes. The future goals will be to understand the molecular details of the trimolecular complex and to probe the subsequent interactions which lead to membrane fusion and virus entry.

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