Substitutions in a Homologous Region of Extracellular Loop 2 of CXCR4 and CCR5 Alter Coreceptor Activities for HIV-1 Membrane Fusion and Virus Entry*

Donald J. Chabot and Christopher C. Broder‡

From the Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814-4799

CXCR4 and CCR5 are the principal coreceptors for human immunodeficiency virus type-1 (HIV-1) infection. Previously, mutagenesis of CXCR4 identified single amino acid changes that either impaired CXCR4's coreceptor activity for CXCR4-dependent (X4) isolate envelope glycoproteins (Env) or expanded its activity, allowing it to serve as a functional coreceptor for CCR5-dependent (R5) isolates. The most potent of these point mutations was an alanine substitution for the aspartic acid residue at position 187 in extracellular loop 2 (ecl-2), and here we show that this mutation also permits a variety of primary R5 isolate Envs, including those of other subtypes (clades), to employ it as a coreceptor. We also examined the corresponding region of CCR5 and demonstrate that the substitution of the serine residue in the homologous ecl-2 position with aspartic acid impairs CCR5 coreceptor activity for isolates across several clades. These results highlight a homologous and critical element in ecl-2, of both the CXCR4 and CCR5 molecules, for their respective coreceptor activities. Charge elimination expands CXCR4 coreceptor activity, while a similar charge introduction can destroy the coreceptor function of CCR5. These findings provide further evidence that there are conserved elements in both CXCR4 and CCR5 involved in coreceptor function.

Seven-transmembrane domain coreceptor molecules are required along with CD4 for human immunodeficiency virus type-1 (HIV-1) envelope glycoprotein (Env)-mediated membrane fusion and virus entry (reviewed in Refs. 1–4). These coreceptors belong or are related to the chemokine receptor subfamily of the seven-transmembrane domain G-protein-coupled receptor superfamily (5). Although more than a dozen related coreceptor molecules have been shown by one or more laboratories to function in the fusion or entry of at least one virus isolate, it is now well recognized that the principal HIV-1 coreceptors remain the initially discovered CXC chemokine receptor CXCR4 and the CC-chemokine receptor CCR5 (6). The viral entry process is believed to occur through a receptor-mediated activation of Env's membrane fusogenic activity. The most favored model for the mechanism underlying this activation process involves at least two stages of receptor-induced conformational alterations in Env; the first is through CD4 binding, leading to exposure of the coreceptor binding site, followed by coreceptor binding and a further presumed conformational change resulting in the exposure and insertion of the hydrophobic fusion peptide domain of the gp41 NH₂ terminus into the receptor-bearing target cell (reviewed in detail in Refs. 7 and 8). Defining the elements of the coreceptor molecules involved in Env interaction and the membrane fusion process remains an area of critical importance for our complete understanding of the virus entry mechanism. To date, a variety of studies, employing the use of both Env and coreceptor genetic chimeras and mutations, have provided an extensive array of important structural information on several coreceptors as well as on Env determinants for coreceptor use, which have been reviewed in detail (3, 7–11). Although not all in complete agreement, these studies have clearly indicated that multiple extracellular domains of the coreceptors are required for supporting the Env-mediated fusion process, involving cooperativity between particular elements of the NH₂-terminal domain and one or more of the three extracellular loops.

We recently studied a large battery of CXCR4 mutants, primarily generated using an alanine-scanning mutagenesis strategy (12). Those results indicated that negatively charged glutamic acid residues in the NH₂ terminus (Glu¹⁴, Glu¹⁵, and Glu²⁵) and the aspartic acid residue (Asp⁹⁷) in extracellular loop 1 (ecl-1) were important for CXCR4 function as a coreceptor for X4 HIV-1 clade B isolates. It was speculated that the role of these negatively charged residues was related to their electrostatic interactions with positively charged Env residues in the V3 loop region of the previously classified syncytium-inducing (X4) Envs (13, 14). In addition, several point mutations, N11A and R30A in the NH₂ terminus and N176A, D187A, and D193A in ecl-2, were noted to enhance the ability of CXCR4 to serve as a coreceptor for otherwise CCR5-dependent R5 HIV-1 isolates, while retaining full function for X4 and R5X4 isolates. The most potent of these mutations was an alanine substitution for the aspartic acid residue at position 187 in ecl-2, which was also identified in another study (15). Additional complexity to the Env-receptor system comes from the assessment of Env regions involved in coreceptor interaction and goes beyond solely a notion of electrostatic interactions between the V3 loop and a coreceptor (recently reviewed in Refs. 8 and 9). More recently, we have discovered that removal of the N-linked glycosylation modifications of the CXCR4 molecule also permit the receptor to serve as a potential coreceptor.
Homologous Region in HIV-1 Coreceptors

23775

for otherwise CCR5-dependent HIV-1 Env s (16). Together these findings support the hypothesis that there are conserved elements important for coreceptor activity between the CXCR4 and CCR5 molecules.

To further investigate the possible existence of important conserved elements in coreceptor activity between the CXCR4 and CCR5 molecules, we focused on the CXCR4-D187A mutant, and here we demonstrate that this mutation also permits a variety of HIV-1 R5 primary isolate Env s, including those of other genetic subtypes, to employ it as a functional coreceptor. Further, we examined the corresponding region of CCR5 and demonstrate that the substitution of the serine residue in the homologous ec1-2 position of CCR5, with aspartic acid, dramatically and specifically impairs CCR5 coreceptor activity for a variety of R5 and R5X4 isolates across several HIV-1 clades. These results highlight a homologous and critical region in ec1-2, of both the CXCR4 and CCR5 major coreceptor molecules, for their respective coreceptor activities. On the one hand, charge elimination can expand CXCR4 coreceptor activity, while a similar charge introduction can destroy CCR5 coreceptor function, depending on the particular isolate Env. The present findings, taken together with other observations, soundly support the hypothesis that conserved extracellular domains, common to both CXCR4 and CCR5 involved in their coreceptor activities exist, and this information will aid the efforts aimed at defining the Env-coreceptor interactions and virus entry process in greater detail.

EXPERIMENTAL PROCEDURES

Cells and Culture Conditions—HeLa cells were obtained from the American Type Culture Collection (Manassas, VA), while human glioblastoma cell line U373-MG and the U373-MG-CX4 derivative cell line were obtained from Adam P. Geller (Harvard School of Public Health, Cambridge, MA) and supplemented with 10% bovine calf serum, 2 mm L-glutamine, and antibiotics (DMEM-15). U373-MG cell monolayers were maintained in Dulbecco's modified Eagle's medium (Quality Biologicals, Gaithersburg, MD) supplemented with 10% bovine calf serum, 2 mm L-glutamine, and antibiotics (DMEM-15). U373-MG cell monolayers were maintained in the same way, except 15% bovine calf serum was used. The U373-MG-CX4 cell monolayers were also supplemented with 200 μg of G418 (Calbiochem)/ml.

Cell lines expressing mutant or wild-type CCR5 molecules were prepared by N-l(3-dioleoyloxypropyl)-N,N,N-trimethylammonium methanesulfate (Roche Molecular Biochemicals) transfection of U373-MG cells with pCDNA3.1 Hygro- (Invitrogen, Carlsbad CA) containing coreceptor gene linked to T7 promoter function, depending on the particular isolate Env. The present findings, taken together with other observations, soundly support the hypothesis that conserved extracellular domains, common to both CXCR4 and CCR5 involved in their coreceptor activities exist, and this information will aid the efforts aimed at defining the Env-coreceptor interactions and virus entry process in greater detail.

PTKA

Plasmids and Recombinant Vaccinia Viruses—For Env expression, we employed a battery of plasmids and recombinant vaccinia viruses encoding the Env genes from several R5, X4, and R5X4 HIV-1 isolates. The following recombinant vaccinia viruses expressing gp160 from different HIV-1 isolates were used: vCB-28 (JR-FL), vCB-32 (SF162), vCB-34 (SP), vCB-39 (ADA), vCB-41 (LAV), vCB-52 (CM235), vCB-53 (CM243) (18), and vDC-1 (89.6) (12). The following recombinant vaccinia viruses expressing gp160 from different SIV isolates were used: vCB-74 (mac239), vCB-75 (mac316), and vCB-76 (mac316mut) (19). Purified vaccinia virus stocks were used at a multiplicity of infection of 10 plaque-forming units/cell. Plasmids encoding gp160 from primary isolates linked to T7 promoter were obtained from the National Institutes of Health AIDS Research and Reagent Program (Rockville, MD) and include 93B0191.10, 93C014.9, 92U0975.10, 93B0299.2, 92TH022–4, MA301985–26, 92BO205–9, HA301593.1, 92TH014–12, 92BO20–4, 91US005.11, 92U0637.8, and 92BO205.5. For CD4 expression, we used recombinant vaccinia virus vCB-3 (20).

Bacteriophage T7 RNA polymerase was produced by infection with vTFI-1 (P11 natural late vaccinia virus promoter) (21). The Escherichia coli lacZ gene linked to the T7 promoter was introduced into cells by infection with vaccinia virus recombinant vCB21R-LacZ, which was described previously (22). For coreceptor expression, we employed recombinant vaccinia virus or one of two alternative plasmid expression protocols. For cell fusion assays, we either infected cells with vaccinia virus encoding chemokine receptor linked to the vaccinia virus pT5 early-late promoter or we transfected cell monolayers with plasmids containing coreceptor genes linked to a strong synthetic vaccinia virus p7.5 early-late promoter (pSv99) (23). Cells were then infected with the Western Reserve wild-type strain of vaccinia virus, and transfection of monolayers was performed with N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate (Roche Molecular Biochemicals). For virus infection assays, cells were transfected with coreceptor genes linked to the cytomegalovirus promoter in pCDNA3 (Invitrogen, Carlsbad CA), and transfection was performed by the DEAE-dextran procedure, as described below.

Mutagenesis—CCR5 and CXCR4 mutations were made by using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) in accordance with the manufacturer's instructions. Two mutagenic polyacrylamide gel electrophoresis-purified oligonucleotides were used per mutation. The identities of all mutant constructs were confirmed by DNA sequencing.

Cell Surface Expression—Wild-type and mutant CCRX4 expression levels were determined by fluorescent antibody staining using the conformation-dependent 12G5 (24) or the conformation-independent 4G10 mouse monoclonal antibodies (mAbs). Target cells were transfected as described above, infected with vCB-3 for 2 h at 37 °C, and incubated overnight in culture medium at 37 °C. Following overnight incubation, monolayers were washed with plates with phosphate-buffered saline (PBS) containing 10 μm EDTA, and cells (0.5–1 x 10⁶) were washed twice with PBS and once with PBS containing 2.5% goat serum on ice. Cells were stained in 100 μl of PBS containing 2.5% goat serum and 2 μg/100 μl 12G5 or 4G10 mAb to CCRX1 for 1 h on ice. Wild-type and mutant CCRX5 expression levels were determined by fluorescent antibody staining using the conformation-dependent 2D7 (25) or 5C7 (26) mAbs. Cells were stained in 100 μl of PBS containing 2.5% goat serum and 2 μg of 2D7 or 5C7 mAb to CCRX5/100 μl for 1 h on ice. Cells were then washed twice with PBS and once with PBS containing 2.5% goat serum. After specific antibody staining, cells were washed and stained in 100 μl of PBS containing 2.5% goat serum and 10 μl of phycoerythrin-labeled goat anti-mouse immunoglobulin G (IgG) for 45 min, washed three times with PBS, and fixed with 2% paraformaldehyde in PBS. Fluorescence was measured with an EPIC XL flow cytometer (Couler, Miami, FL).

Cell Fusion Assays—Fusion between Env-expressing and receptor-expressing cells was measured using a reporter gene assay in which the cyttoplasm of one cell population contained vaccinia virus-encoded T7 RNA polymerase and the cyttoplasm of the other contained the E. coli lacZ gene linked to the T7 lac promoter; β-galactosidase is synthesized in fused cells (27). Cell fusion reactions were conducted with the various indicated cell mixtures in 96-well plate format at 37 °C. Typically, the ratio of CD4-expressing to Env-expressing cells was 1:1 (2 x 10⁶ total cells/well, 0.2-ml total volume). Cytosine arabinoside (40 μg/ml) was added to the fusion reaction mixture to reduce nonspecific β-galactosidase production (28). For quantitative analyses, nonlabeled P-40 was added (0.5% final concentration) at 3 h, and aliquots of the lysates were assayed for β-galactosidase at ambient temperature with the substrate chlorophenol red-β-galactopyranoside (Roche Molecular Biochemicals). Fusion results were calculated and expressed as rates of β-galactosidase activity (change in optical density at 570 nm/min x 1000) (27), and all fusion reactions and β-galactosidase quantifications were performed in duplicate.

HIV-1 Infection Studies—U373-MG-CX4 target cells were prepared in 48-well plates and transfected with the desired coreceptor-encoding plasmid by the DEAE-dextran method. Briefly, 0.2 μg of DNA mixed in 110 μl of DMEM-2.5 with 100 μl chloroquine diphosphate, and 1.1 μl of DEAE-dextran stock (10 mg/ml) was added to each well of semiconfluent cells. After 4 h, medium was replaced with 10% MeSO/FPBS for 2 min. Monolayers were then washed with PBS and incubated overnight in DMEM-15. Viral infection assays were performed with a luciferase reporter HIV-1 Env pseudotyping system (29). Viral stocks were prepared as described previously by transfecting 293T cells with plasmids encoding the luciferase virus backbone (pNL-Luc-Er) and Env from HIV strain 89.6 (30), JR-FL (31), NLA–3 (LAV) (32), (33), or SF2 (34). The resulting supernatant was clarified by centrifugation for 10 min at 7500 g in a Sorval RT-7 centrifuge (RTH-750 rotor) and stored at 4 °C. Monolayers were infected with 100 μl of virus containing 8 μg/ml DEAE-dextran. After 2 h, 0.5 ml of DMEM-15 was added to each well. Cells were lysed at 72 h postinfection by resuspension in 105 μl of cell lysis buffer (Promega, Madison, WI), and 50 μl of the resulting supernatant was assayed for luciferase activity. The luciferase activity was measured in a luminometer. The results were expressed as luciferase units per 10⁶ cells per mg DNA. The data are representative of at least two experiments.
RESULTS

Charge Deletion in ecl-2 and the Expansion of CXCR4 Co-receptor Activity—Previously, we demonstrated that several alanine substitution point mutations in CXCR4 (N11A, R30A, D187A, D193A) either alone or in combination could allow CXCR4 to serve as a functional coreceptor for prototypic R5 HIV-1 clade B Envs while retaining its coreceptor activity for X4 and R5X4 HIV-1 clade B Envs (12). Combining some of these single mutations together resulted in greater than additive effects on expanding CXCR4 coreceptor activity. The single most potent coreceptor-expanding mutation was the elimination of the negatively charged aspartic acid residue at position 187 (Asp^{187}) in ecl-2. We speculated that the removal of this key negatively charged residue in ecl-2 reduced the domain's net negative charge and allowed for an appropriate region of an R5 Env, perhaps including the V3 loop, to properly associate with the altered coreceptor's extracellular surface, which is otherwise repelled, and results in Env-mediated fusion. Similar results concerning the Asp^{187} residue were also reported by Wang et al. (15). The expansion of CXCR4’s coreceptor activity, to include its recognition and use by prototypic clade B R5 HIV-1 isolate Envs, as a result of just a single charge-deleting point mutation was somewhat surprising. This observation strongly suggested the presence of conserved elements, between the principal coreceptors CXCR4 and CCR5, important for Env interaction and their coreceptor activity. To investigate this observation further and determine the breadth of the mutation’s effect and whether there is a similar important site in CCR5, we performed a series of additional experiments examining primary isolate HIV-1 Envs and additional CCR5 mutants.

We employed the well characterized β-galactosidase reporter gene assay for HIV-1 Env-mediated cell fusion in our analysis (18, 27, 35). In these experiments, plasmids encoding wild-type CXCR4 or CCR5 and the CXCR4-D187A mutant under control of a vaccinia virus promoter were transfected into U373-MG cells, and CD4 was supplied by infection with a recombinant vaccinia virus. Shown in Fig. 1A is the coreceptor activity of CXCR4-D187A, in comparison with wild-type CXCR4 and CCR5, for a variety of prototypic CCR5-dependent R5 HIV-1 clade B isolate Envs. The surface-expressed level of the CXCR4-D187A mutant was equivalent to that of wild-type CXCR4 as determined by either 12G5 and 4G10 mAb immunostaining and flow cytometry (Ref. 12 and data not shown). The level of coreceptor activity of CXCR4-D187A, for these otherwise CCR5-dependent Envs, was significant and ranged from 36 to 69% of the fusion signals obtained with wild-type CCR5 in the same experiment (Fig. 1A). The actual rate of reporter gene activity is shown along with the level of activity obtained with a mock-transfected (vector) cell population expressing CD4 without any coreceptor for comparison. In other experiments, the CXCR4-D187A molecule could also serve as a functional coreceptor in virus entry assays using the HIV-1 luciferase reporter gene pseudotypes system (29) with the R5 Envs JR-FL, ADA, Ba-L, and SF162 (12). Because this single amino acid change could so dramatically expand CXCR4’s coreceptor activity for prototypic R5 clade B Envs, we examined additional R5 HIV-1 primary isolate Envs including those of alternate genetic subtypes (clades) to determine whether they could employ it as a functional coreceptor as well.

A panel of plasmid-encoded primary isolate Envs were employed in the cell fusion assay and screened for their ability to utilize the CXCR4-D187A mutant in comparison with wild-type CXCR4 and CCR5. Two primary clade B isolates (91US005.11 and 92BR020.4), a clade F/B mosaic (93BR019.10-all gp120 F), and a clade A (92RW020.5) R5 Env were clearly quite capable of employing the CXCR4-D187A mutant coreceptor, having Env fusion activities nearly equivalent to the level of activity obtained with target cells expressing wild-type CCR5 (Fig. 1B). The X4 Env LAV was also included as a control in this experiment as in Fig. 1A, to confirm that wild-type CXCR4 and the D187A mutant were equivalent in expression and function (data not shown). The overall reporter gene signals were lower in this experiment in comparison with Fig. 1A because both the coreceptor genes and the Env genes (most driven by a T7 promoter system) were transfected as plasmids in this assay (Fig. 1B). Although not universally accepted as a functional coreceptor for all R5 Envs examined, the CXCR4-D187A mutant clearly supported Env-mediated fusion for additional isolates other than those exclusively from clade B.
Charge Introduction into ecl-2 and the Impairment of CCR5 Coreceptor Activity—We recently reported that the removal of the N-linked glycosylation sites in CXCR4 could allow the protein to serve as a universal coreceptor for both X4 and R5 laboratory-adapted and primary HIV-1 strains (16). We hypothesized that this alteration unmasked existing common extracellular structures, reflecting a conserved three-dimensional similarity of important elements between CXCR4 and CCR5 that are involved in HIV-1 Env interaction. Also in support of this notion, we have observed that the mouse monoclonal antibody 12G5, which binds to elements of the ecl-2 of CXCR4, inhibited R5 Env-mediated fusion with the D187A mutant CXCR4 coreceptor (data not shown). These observations, together with the fusion results obtained with CXCR4-D187A, suggest that there is an existing underlying conserved framework of elements between CXCR4 and CCR5 for their coreceptor activity and prompted us to examine the homologous region in ecl-2 of the CCR5 coreceptor corresponding to the Asp187 position of CXCR4. We performed an alignment of the ecl-2 regions of CXCR4 and CCR5 (Fig. 2) and noted that a pair of serine residues were located in CCR5 at the position corresponding to Asp187 in CXCR4. Two mutant CCR5 constructs were prepared; one was a double alanine substitution of both serine residues at positions 179 and 180 (S179A/S180A), which would be the direct opposite of the present CCR5-S179D mutation. Two mutant CCR5 constructs were prepared; one was a double alanine substitution of both serine residues at positions 179 and 180 (S179A/S180A), which would be the direct opposite of the present CCR5-S179D mutation. We hypothesized that this alteration unmasked existing common extracellular structures, reflecting a conserved three-dimensional similarity of important elements between CXCR4 and CCR5 that are involved in HIV-1 Env interaction. Also in support of this notion, we have observed that the mouse monoclonal antibody 12G5, which binds to elements of the ecl-2 of CXCR4, inhibited R5 Env-mediated fusion with the D187A mutant CXCR4 coreceptor (data not shown). These observations, together with the fusion results obtained with CXCR4-D187A, suggest that there is an existing underlying conserved framework of elements between CXCR4 and CCR5 for their coreceptor activity and prompted us to examine the homologous region in ecl-2 of the CCR5 coreceptor corresponding to the Asp187 position of CXCR4. We performed an alignment of the ecl-2 regions of CXCR4 and CCR5 (Fig. 2) and noted that a pair of serine residues were located in CCR5 at the position corresponding to Asp187 in CXCR4. Two mutant CCR5 constructs were prepared; one was a double alanine substitution of both serine residues at positions 179 and 180 (S179A/S180A), and the other was a substitution of the serine residue at position 179 with aspartic acid (S179D). Asp187 in CXCR4 was investigated with a D187K mutation by Wang et al. (15), and this alteration had only moderate inhibitory effects on CXCR4 coreceptor activity for the X4 isolate IIIB and had no effect on enhancing utilization by two R5 Envs (Ba-L and ADA). In addition, a D187S mutation was examined (15), which is the wild-type or mutant CCR5 genes. Clones surviving hygromycin treatment (200 µg/ml) were isolated and expanded. Cell surface expression of CCR5 was determined by immunostaining with CCR5 mAb 5C7 followed by staining with phycoerythrin anti-mouse IgG. Mean fluorescence intensities were determined with a flow cytometer (model XL-MCL; Coulter). Mean fluorescence intensity values for cell lines are compared with U373-MG-CD4+/CCR5 wild-type cells, which are regarded as 100%, and U373-MG-CD4+ cells, which are regarded as 0%. The solid line represents U373-MG-CD4+ parent cells. The dashed line represents U373-MG-CD4+/CCR5 wild-type cells. The dotted line represents U373-MG-CD4+/CCR5 S179A/S180A cells (Mean fluorescence intensity = 94%). The mixed line represents U373-MG-CD4+/CCR5-S179D cells (Mean fluorescence intensity = 75%). Similar results were obtained with anti-CCR5 mAb 2D7 (data not shown).

Although the recombinant Env-based cell fusion assay is certainly a reliable system to study the HIV-1 Env-mediated membrane fusion process, including the examinations of other Env mutants or mutations in their cellular receptors, we wished to further confirm the present cell fusion findings with an additional system. Therefore, we examined these mutant CCR5 coreceptors for their ability to provide coreceptor function in a virus entry assay by employing the luciferase reporter.
gene HIV-1 Env pseudotyping system (29). Viral stocks were prepared as described previously by transfecting 293T cells with plasmids encoding the luciferase virus backbone (pNL-Luc-E’ R’-) and Env from HIV-1 strains JR-FL, SF2, or 89.6. HIV-1 luciferase reporter pseudovirus-containing supernatants were clarified by centrifugation and stored in 30% calf serum at −80 °C. For reporter-virus infection, target cells were prepared in 48-well plates in triplicate. We observed results similar to the cell fusion data, where both CCR5 mutant constructs had approximately wild-type levels of coreceptor activity with the R5 JR-FL Env, while the single CCR5-S179D mutant was significantly defective in providing coreceptor function for both the R5X4 Envs 89.6 and SF2, exhibiting 1–2 logs less activity in comparison with wild-type CCR5. The clade E R5 Env CM235 plasmid clone was not suitable for HIV-1 pseudovirus construction. In addition, neither the single (S179D) nor double (S179A/S180A) CCR5 mutant enhanced any X4 HIV-1 Env-mediated fusion or virus entry (data not shown). Thus, this homologous position in ecl-2 of both CXCR4 and CCR5 appears important for coreceptor activity and is quite sensitive to alteration depending on the particular Env examined.

**Charge Introduction into ecl-2 and the Impairment of CCR5 Coreceptor Activity for Primary Isolate R5 Env-mediated Fusion**—Because of the dramatic effect of the S179D mutation in CCR5 in abolishing fusion by the R5 CM235 Env, we further examined both CCR5 mutants for their ability to support Env-mediated fusion with an expanded battery of primary isolate R5 Envs, including those from alternate HIV-1 clades. In this experiment, as in the cell fusion experiment with primary isolate Envs shown in Fig. 1B, the battery of Env clones was expressed using a plasmid transfection protocol where the Env genes were driven by the T7 promoter system. Only a limited number of cloned functional primary isolate R5 Envs were available for testing. Transfections were also performed with plasmids encoding the CM235 and Ba-L Envs for comparison, but these were expressed by a vaccinia virus promoter. The level of reporter gene activity with the vector only containing control cell line is also shown for comparison (Fig. 6). We observed that the inhibitory effect of the S179D mutation in CCR5 was evident across several of these clades (Fig. 6). Consistent with the results obtained with the prototypic clade B R5 Envs (JR-FL and ADA), the CCR5-S179D mutant had near equivalent coreceptor activity with the prototypic clade B R5 Env Ba-L and with two of four primary isolate clade B R5 Envs (Fig. 6). However, there were two clade B Envs that exhibited some difficulty in employing the CCR5-S179D coreceptor; the HA301593.1 clade B R5 Env was fusion-negative with CCR5-S179D, while another (91US005.11) had fusion activity reduced by ~50% when compared with wild-type CCR5. The inhibitory effects of the S179D CCR5 mutation were observed with somewhat more consistency with Envs across the other HIV-1 clades, where the Env-mediated fusion was either completely abolished or inhibited anywhere from 50 to 75%. Two clade F/B mosaics (93BR019.10 and 93BR019.4), both of which are all gp120 of clade F, and the clade F isolate Env 93BR029.2 were all significantly inhibited in an ability to utilize the CCR5-S179D mutant. The CCR5-S179D mutant was also defective in supporting fusion mediated by the clade G R5 Env (92UG975.10). Of a pair of Envs examined from clades A and C, fusion was either abolished as with isolates 92RW020.5 (A) and MA301965.26 (C) or significantly reduced (~50%) as with isolates 92UG037.8 (A) and 92BR025.9 (C). Interestingly, an additional clade E R5 Env (92TH022.4) appeared unaffected by the S179D CCR5 mutation, while the CM235 clade E Env was again unable to employ the CCR5-S179D mutant as a functional coreceptor. In a separate experiment, an additional clade E R5 Env (CM243) was also found incapable of utilizing the CCR5-S179D mutant (data not shown). In no instances did the (S179A/S180A) CCR5 mutant appear unable to support fusion by any of the Envs examined. Thus, in the majority of cases, this single charge-introducing mutation could completely disrupt CCR5 coreceptor activity for a variety of R5 HIV-1 primary isolate Envs across several alternative clades.

**DISCUSSION**

HIV-1 is a complex retrovirus that has uniquely evolved to use a dual receptor system to infect appropriate host cells, unlike any other known virus. The entry of HIV-1 is a pH-independent Env-mediated membrane fusion process thought to occur through a two-step receptor-induced mechanism. Presently, the most favored model for this process describes an initial binding of Env to CD4, which induces conformational alterations in the gp120 subunit, leading to the exposure of a conserved coreceptor binding site. Following coreceptor binding, secondary conformational changes occur in Env, triggering...
the fusion activity of the gp41 subunit (reviewed recently in Refs. 8 and 36). The delineation of the critical regions involved in the interactions within the Env-CD4-coreceptor complex and their relationship to the mechanism of the fusion process remain an area of intensive investigation. In principal, this is because a detailed understanding of this process will lead to new insights for the development of molecular-based intervention strategies and facilitate the development of new vaccine designs (37, 38). Until such an advance as a structural solution of an Env-CD4-coreceptor complex, mutagenesis and the analysis of its consequences remains a reliable approach toward deciphering the determinants of this intricate system.

Recently, much of the focus in this area has been devoted to defining the important elements in the coreceptors responsible for their activity in virus entry and chemokine ligand binding. To date, the consensus of many reports is that multiple extracellular elements or domains of the coreceptors are involved in Env interaction and that these interactions are complex, where individual Env s may interact with the same coreceptor in alternative ways (reviewed in detail in Refs. 2 and 9–11). By using a large number of mutants, chimeras, and homologs of CCR5, it has been convincingly demonstrated that the NH2 terminus plays a critical role in membrane fusion and in the entry of R5 HIV-1 and M-tropic simian immunodeficiency virus (SIV) isolates (19, 39–48). More recently, additional studies on the CCR5 extracellular regions involved in Env interaction and chemokine ligand binding, combined with epitope mapping of panels of CCR5 mAbs, have generated interesting and somewhat unexpected findings (49, 50). NH2-terminal reactive antibodies versus those directed against epitopes composed of elements of ecl-2 of CCR5 were examined for their ability to block gp120 or chemokine binding and HIV-1 infectivity. These independent studies noted no correlation between a mAb's ability to block gp120 binding with an ability to prevent Env-mediated membrane fusion and virus infection. Both studies noted that CCR5 ecl-2-reactive mAbs were more potent inhibitors of virus infection than those directed primarily to the NH2 terminus of the molecule, while mAbs to the CCR5 NH2 terminus blocked gp120 binding more effectively than those directed against ecl-2. Together, these observations have strongly implicated the NH2 terminus in interacting directly with gp120, while the extracellular loops were also important, perhaps involved in inducing conformational changes in Env, and indicated that the gp120 and chemokine binding sites of CCR5 were distinct yet overlapping. Similar findings have also been shown with CXC4 coreceptor functional domains and the binding sites for its chemokine ligand stromal cell-derived factor-1 (51).

Initial studies on the functional analysis of CXC4R employed the use of small truncations in either the amino or carboxyl termini and genetic chimeras. The NH2 terminus was the first domain proposed to play a role in coreceptor function from studies showing NH2-terminal reactive polyclonal antibody blocking of fusion and virus entry (35). A second report demonstrated that the NH2 terminus was indeed critical for some isolates yet not the sole element deemed important (52), while additional studies highlighted the importance of other domains, primarily ecl-2, in coreceptor activity (53, 54). We have recently shown an importance of negatively charged glutamic acid residues in the NH2 terminus, as well as some additional charged residues in the extracellular loops, for X4 Env coreceptor activity (12). Surprisingly, the removal of the negatively charged aspartic acid residue at position 187 by an alanine substitution in ecl-2 was noted to expand CXC4R's coreceptor activity, allowing support of laboratory-adapted R5 HIV-1 Env-mediated fusion and virus infection while having no effect on X4 Env usage (12, 15). Taken together, these observations suggested that there were existing conserved elements between CXCR4 and CCR5 for their respective coreceptor activities, and they prompted us to further examine the CXCR4-D187A mutation with respect to a broader range of R5 HIV-1 primary isolates and to examine the homologous region in ecl-2 of the CCR5 coreceptor corresponding to the Asp187 position of CXCR4.

Indeed, in addition to the NH2 terminus of the coreceptors, the second region of functional importance includes the extracellular loops (45, 46, 54, 55). The ecl-2 appears to be the most critical of the three extracellular loops and is involved in coreceptor activity (25, 41, 46, 56), interaction with the gp120-CD4 complex (25) and chemokine binding (50, 57). For example, the CCR5 mAb 2D7, mapped to ecl-2 (50, 58), was able to inhibit binding of the JR-FL gp120-CD4 complex to CCR5, efficiently block the entry of R5 (M-tropic) and R5X4 (dual-tropic) HIV-1 strains, inhibit the binding of chemokine ligands to CCR5 (25), and also inhibit the co-immunoprecipitation of CD4 and CCR5 (59). In comparison, the CXC4R mAb 12G5, which can block the fusion and entry of X4 and R5X4 HIV-1 strains, maps to CXC4R ecl-2 and also blocks chemokine (stromal cell-derived factor-1) binding (53, 60, 61). Clearly, the ecl-2 of both CXC4R and CCR5 is a critical loop domain for their respective coreceptor activities and natural ligand binding abilities.

In regard to the mutations that confer on, or reveal in, CXC4R an ability to be employed as a coreceptor by otherwise CCR5-dependent HIV-1 Env s, it was the CXCR4-D187A ecl-2 mutation that had the most potent effect. Although it was the clade B R5 isolates, both laboratory-adapted and primary, that were most capable of utilizing CXCR4-D187A, there were some other primary isolate R5 Env s from additional clades that could as well (Fig. 1), including clades A and F. We suggest that the removal of the negatively charged aspartic acid residue in ecl-2 of CXCR4 enhances its interaction with R5 Env s. However, an alternative interpretation of this data is that this ecl-2 alteration may change the way CXCR4 associates with CD4, perhaps resulting in a configuration more like the constitutive CCR5-CXCR4 interaction (59), allowing R5 HIV-1 Env s to recognize the receptors in a fashion conducive to membrane fusion. We are exploring both of these possibilities. Also of note is that CXCR4 is the principal receptor for the related feline immunodeficiency virus retrovirus (62), and whereas the D187A CXCR4 mutation can expand coreceptor activity for HIV-1 R5 Env s, this alteration completely ablated the ability of feline immunodeficiency virus to employ it as its primary receptor (63). Feline immunodeficiency virus uses CXCR4 without CD4, and perhaps its sensitivity to the removal of this negatively charged residue in ecl-2 reflects a greater dependence on negatively charged elements in a CD4-independent Env-receptor system. It would be of interest to examine whether the described CD4-independent variants of HIV-1 (64, 65) might also be sensitive to the removal of Asp187 in the CXCR4 coreceptor, since ecl-2 of CXCR4 appears critical for CD4-independent use by HIV-2 (66).

Conversely, the mutation of the serine residue at the homologous position 179 in CCR5 ecl-2 (Fig. 2) by the introduction of the aspartic acid residue could dramatically inhibit the molecule’s ability to support fusion and virus infection by some R5 and R5X4 HIV-1 isolates (Figs. 4 and 5). This mutation maps near the 2D7 epitope but had no effect on 2D7 reactivity as measured by cell surface binding (Fig. 3). This position in CCR5 ecl-2 was also noted in a study of human/mouse CCR5 chimeras and point mutants that revealed a requirement for multiple extracellular domains in coreceptor activity (56) and also that a hydrophobic substitution of the serine residue at position 180
in human CCR5 with proline (the corresponding murine residue) reduced coreceptor activity by ~80%. However, effects on the conformation of ecl-2 as a result of this mutation were not addressed.

There are several possible explanations for how the S179D CCR5 mutation affects the Env-CD4-coreceptor interaction required for the membrane fusion process. First, and perhaps most obvious, would be that this CCR5 mutant is defective in Env binding. Although the majority of evidence implicates the NH2-terminal region in the CCR5-gp120 interaction, as discussed above, the involvement of ecl-2 in binding cannot be ruled out. An alternative speculation that might be made is that this mutation disrupts the CCR5-CD4 interaction, but both of these notions seem less likely, since some R5 Env s are still capable of employing CCR5-S179D. The single S179D mutation, which introduces the aspartic acid residue present at the homologous position in CXCR4, is possibly disrupting CCR5’s ability to correctly associate with certain Envs (Fig. 5), perhaps those that have a greater dependence on CCR5 ecl-2 for triggering fusion activity. Taken together, it is the ecl-2 of both of the principal coreceptors that appears to play an important role in defining coreceptor function and their complex structure.

Indeed, coreceptor conformation appears quite important for function. In fact, different conformational states of CCR5 have been observed in a cell type-dependent manner as determined by mAb reactivity (40, 50). Also, the CXCR4 mAb 12G5 has been shown to differentially inhibit HIV-1 infection in both a cell type- and virus strain-dependent manner (60) suggesting that CXCR4 could also be differentially processed. It has also been suggested that a high molecular weight species of CXCR4, defective in coreceptor activity, is present in human macrophages and that post-translational modifications could account for this observation (67). Structural and/or conformational alterations such as these might certainly effect an Env’s ability to utilize a particular coreceptor molecule. Directed mutagenesis of CXCR4 and CCR5 targeting the pairs of likely disulfide bonds in the extracellular domains has also supported this view, and it appears that both predicted disulfide bonds are present and that their disruption can have moderate to severe consequences on the molecules structure as detected by conformation-dependent mAb binding and coreceptor activity (12, 68, 69).

An important difference between HIV-1 and SIV is that all SIV strains studied to date appear highly specific for CCR5 regardless of their cellular tropism (T-tropic versus M-tropic).
in the development of therapeutics to block HIV-1 that target when their effects on R5 Env usage are examined across alterations that exist between Envs and coreceptors, especially homologous ecl-2 alterations also highlight the differential interactions that exist between Envs and coreceptors, especially across isolates. The present data concerning the effects of these ho-

acid residue dramatically inhibited the molecule’s ability to exist across all HIV-1 clades. These new findings may prove helpful in efforts to understand the interactions between HIV-1 Env and receptors and in efforts to block these associations, which lead to infection.

**Acknowledgments**—We thank Joseph Isaac for viruses and cells. Plasmids for pseudovirus construction were generously provided by Robert Doms (University of Pennsylvania). Monoclonal antibodies 2G7 and 5C7 were generously provided by Lijun Wu. A number of reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health.

**REFERENCES**

1. Dimitrov, D. S., Xiao, X., Chabot, D. J., and Broder, C. C. (1998) *J. Membr. Biol.* 165, 70–90.

2. Clapham, P. R., Reeves, J. D., Simmons, G., Dejucq, N., Hibiitsu, S., and McKnight, A. (1999) *Mem. Biol.* 16, 49–60.

3. Moore, J. P., Takan, A., and Desjarlais, T. (1997) *Curr. Opin. Immunol.* 9, 551–562.

4. Doms, R. W., and Peiper, S. C. (1997) *Virology* 235, 179–190.

5. Murphy, P. M. (1996) *Cytokine Growth Factor Rev.* 7, 47–64.

6. Berson, J. F., and Jones-Trower, A. (1999) in *Human Retroviruses and AIDS* (Korber, B., Foley, B., Leitner, T., McCutchan, F., Hahn, B., Mellors, J. W., Myers, G., and Kuilen, C., eds) Vol. III, pp. 20–45, Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, Los Alamos, NM.

7. Dimitrov, D. S., and Broder, C. C. (1997) *HIV and Membrane Receptors*, Landes Bioscience, Austin, TX.

8. Berger, E. A., Murphy, P. M., and Farber, J. M. (1999) *Annu. Rev. Immunol.* 17, 657–700.

9. Hoffman, T. L., and Doms, R. W. (1999) *Mem. Biol.* 16, 57–65.

10. Bieniasz, P. D., and Cullen, B. R. (1998) *Front. Biosci.* 3, D44–D58.

11. Berson, J. F., and Doms, R. W. (1998) *Semin. Immunol.* 10, 237–248.

12. Chabot, D. J., Zhang, F. P., Quinnan, G. V., and Broder, C. C. (1999) *J. Virol.* 73, 6598–6609.

13. De Jong, J. J., De Jonge, D. A., Kostert, W., and Mols, M., and Goudsmit, J. (1992) *J. Virol.* 66, 6777–6780.

14. Fouchier, R. A., Groenink, M., Kootstra, N. A., Termesiste, M., Huisman, H. G., Miedema, F., and Schuitemaker, H. (1992) *J. Virol.* 66, 3183–3187.

15. Wang, Z., Berson, J. F., Zhang, T., Chen, Y.-H., Sun, Y., Sharron, M., Lu, Z., and Peiper, S. C. (1998) *J. Biol. Chem.* 273, 15007–15015.

16. Chabot, D. J., Chen, H., Dimitrov, D. S., and Broder, C. C. (2000) *J. Virol.* 74, 4404–4413.

17. Harrison, E. D., and Gehrke, A. P. (1993) *J. Virol.* 67, 5397–5397.

18. Broder, C. C., and Berger, E. A. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 9004–9008.

19. Edinger, A. L., Amedee, A., Miller, K., Doranz, B. J., Endres, M., Sharron, M., Samson, M., Lu, Z. H., Clements, J., Murphy, C., Merrieh, M., Peiper, S. C., Parmentier, M., Broder, C. C., and Doms, R. W. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 94, 4005–4010.

20. Broder, C. C., Dimitrov, D. S., Blumenthal, R., and Berger, E. A. (1993) *Virology* 193, 483–491.

21. Alexander, W. A., Moss, B., and Farber, T. R. (1992) *J. Virol.* 66, 2934–2942.

22. Leal, D. G., Broder, C. C., and Berger, E. A. (1996) *J. Virol.* 70, 5487–5494.

23. Chakrabarti, S., Sier, J. R., and Moss, B. (1995) *BioTechniques* 23, 1094–1097.

24. Szezaki, J. M., Turner, D. J., Collman, R. G., Hoxie, J., and Gonzalez-Scarano, F. (1997) *J. Virol.* 71, 5678–5683.

25. Wu, L., LaRose, G., Kassam, N., Gordon, C. J., Heath, H., Ruffing, N., Chen, H., Humblis, J., Samson, M., Parmentier, M., Moore, J. F., and Mackay, T. C. (1997) *J. Exp. Med.* 185, 737–743.

26. Leal, D. G., Wu, L., Hoxie, J. A., Springer, T. A., and Mackay, C. R. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 1925–1930.

27. Nussbaum, O., Broder, C. C., and Berger, E. A. (1994) *J. Virol.* 68, 5411–5422.

28. Berger, E. A., Nussbaum, O., and Broder, C. C. (1995) in *HIV: A Practical Approach* (Karn, J., ed) Vol. 2, pp. 125–145, Oxford University Press, Cambridge.

29. Connor, R. I., Chen, B. K., Choe, S., and Landau, N. R. (1995) *Virology* 206, 935–944.

30. Collman, R., Balliet, J. W., Gregory, S. A., Friedman, H., Keislin, D. L., St. John, M. A., and St. John, N. (1992) *J. Virol.* 66, 7291–7297.

31. O’Brien, W. A., Koyanagi, Y., Namaize, A., Zhao, Q. J., Diagne, A. I., and Dack, J. A., and Chen, S. Y. (1990) *Nature* 348, 69–73.

32. Adachi, A., Gendelman, H. E., Koegn, S., Folks, E. E., Willey, R., and Martin, M. A. (1986) *J. Virol.* 59, 284–291.

33. Quinlan, G. V. Jr., Zhang, F. P., Fu, D. W., Dong, M., and Margolick, J. B. (1998) *AIDS Res. Hum. Retroviruses* 14, 95–99.

34. Sanchez-Pescador, R., Powner, M. D., Bar, J. P., Steiner, K. S., Stempf, M. M., Brown-Shimer, S. L., Goe, W. W., Renard, A., Randolph, A., Levy, J. A., Dina, D., and Lavire, P. A. (1985) *Science* 227, 484–492.

35. Feng, Y., Broder, C. C., Kennedy, P. E., and Berger, E. A. (1996) *Science* 272, 872–877.

36. Wyatt, R., and Rodnicki, J. (1988) *Science* 200, 1844–1888.

37. Chen, D. C., and Kim, P. S. (1990) *Cell* 63, 681–684.

38. LaCasse, R., Follis, K. E., Trahey, M., Scarborough, J. D., Littman, D. R., and Nuneberg, J. H. (1999) *Science* 283, 357–362.

39. Wang, Z., Lee, B., Murray, J. L., Bonneau, F., Sun, Z., Schweickart, V., Zhang, T., and Peiper, S. C. (1999) *J. Biol. Chem.* 274, 24314–24319.

40. Hill, C. M., Kwon, D., Jones, M. E., Babson, M., Daughtery, B. L., DeMartino, J. A., Springer, M. S., Unutmaz, D., and Littman, D. R. (1998) *Virology* 249, 357–371.
Homologous Region in HIV-1 Coreceptors

41. Doranz, B. J., Lu, Z. H., Rucker, J., Zhang, T. Y., Sharron, M., Cen, Y. H., Wang, Z. X., Guo, H. H., Du, J. G., Accavitti, M. A., Doms, R. W., and Peiper, S. C. (1997) J. Virol. 71, 6295–6314
42. Dragic, T., Trkola, A., Lin, S. W., Nagashima, K. A., Kajumo, F., Zhao, L., Olson, W. C., Wu, L., Mackay, C. R., Allaway, G. P., Sakmar, T. P., Moore, J. P., and Maddon, P. J. (1998) J. Virol. 72, 279–285
43. Farzan, M., Choe, H., Vaca, L., Martin, K., Sun, Y., Desjardins, E., Ruffing, N., Wu, L., Wyatt, R., Gerard, N., Gerard, C., and Sodroski, J. (1998) J. Virol. 72, 1160–1164
44. Farzan, M., Choe, H., Martin, K. A., Sun, Y., Sidlko, M., Mackay, C. R., Gerard, N. P., and Dragic, T. (1998) J. Virol. 72, 6854–6857
45. Atchison, R. E., Gosling, J., Montecarlo, F. S., Franci, C., Digilio, L., Chao, I. F., and Goldsmith, M. A. (1996) Science 274, 1924–1926
46. Bieniaz, P. D., Fridell, R. A., Aramori, I., Ferguson, S. S., Caron, M. G., and Cullen, B. R. (1997) EMBO J. 16, 2599–2609
47. Rabut, G. E., Konner, J. A., Kajumo, F., Moore, J. P., and Dragic, T. (1998) J. Virol. 72, 3464–3468
48. Picard, L., Simmons, G., Power, C. A., Meyer, A., Weiss, R. A., and Clapham, P. R. (1997) J. Virol. 71, 5003–5011
49. Olson, W. C., Rabut, G. E., Nagashima, K. A., Tran, D. N., Anselma, D. J., Monard, S. P., Segal, J. P., Thompson, D. A., Kajumo, F., Gou, Y., Moore, J. P., Maddon, P. J., and Dragic, T. (1999) J. Virol. 73, 4145–4155
50. Lee, B., Sharron, M., Blanpain, C., Doranz, B. J., Vakili, J., Setoh, P., Berg, E., Liu, G., Hoxie, J. A., Peiper, S. C., Brass, L. F., and Doms, R. W. (1999) J. Virol. 73, 2752–2761
51. Brelot, A., Heveker, N., Lee, B., Vakili, J., Doranz, B. J., Govaerts, C., Migeotte, I., Picard, L., and Dragic, T. (1999) J. Virol. 73, 2721–2730
52. Picard, L., Wilkinson, D., McNeill, A., Gray, P., Hoxie, J., Clapham, P., and Weiss, R. (1997) Virology 231, 105–111
53. Brelot, A., Heveker, N., Pleskoff, O., Sol, N., and Alizon, M. (1997) J. Virol. 71, 4744–4751
54. Lu, Z., Berson, J. F., Chen, Y., Turner, J. D., Zhang, T., Sharron, M., Jenkins, M. H., Wang, Z., Kim, J., Rucker, J., Hoxie, J. A., Peiper, S. C., and Doms, R. W. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6426–6431
55. Rucker, J., Samson, M., Doranz, B. J., Libert, F., Berson, J. F., Broder, C. C., Vassart, G., Doms, R. W., and Parmentier, M. (1996) Cell 87, 437–446
56. Ross, T. M., Bieniaz, P. D., and Cullen, B. R. (1998) J. Virol. 72, 1918–1924
57. Samson, M., LaRosa, G., Libert, F., Paindavoine, P., Dethieux, M., Vassart, G., and Parmentier, M. (1997) J. Biol. Chem. 272, 24934–24941
58. Siciliano, S. J., Kuhmann, S. E., Wang, Y., Madani, N., Springer, M. S., Lineberger, J. E., Danzeisen, R., Miller, M. D., Kavanagh, M. P., DeMartino, J. A., and Kahet, D. (1999) J. Biol. Chem. 274, 1905–1913
59. Xiao, X., Wu, L., Stantchev, T. S., Feng, Y. R., Ugolini, S., Chen, H., Shen, Z., Riley, J. L., Broder, C. C., Sattentau, Q. J., and Dimitrov, D. S. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 7496–7501
60. McIntosh, A., Wilkinson, D., Simmons, G., Talbot, S., Picard, L., Abuja, M., Marsh, M., Hoxie, J. A., and Clapham, P. R. (1997) J. Virol. 71, 1692–1696
61. Hesselgesser, J., Liang, M., Hoxie, J., Greenberg, M., Bras, L. F., Orsini, M. J., Taub, D., and Horuk, R. (1996) J. Immunol. 160, 877–883
62. Willett, B. J., Picard, L., Hoxie, J. A., Turner, J. D., Adema, K., and Clapham, P. R. (1997) J. Virol. 71, 6407–6415
63. Breitman, R., Heveker, N., Adema, K., Hoxie, J. A., Willett, B., and Alizon, M. (1999) J. Virol. 73, 2576–2586
64. Kolchinsky, P., Mirzabekov, T., Farzan, M., Kiprilov, E., Cayabyab, M., W. A., Choe, H., and Sodroski, J. (1999) J. Virol. 73, 8120–8126
65. Hoxie, J. A., LaBranche, C. C., Endres, M. J., Turner, J. D., Berson, J. F., Doms, R. W., and Matthews, T. J. (1998) J. Reprod. Immunol. 41, 197–211
66. Reeves, J. D., Heveker, N., Brolet, A., Alizon, M., Clapham, P. R., and Picard, L. (1998) J. Gen. Virol. 79, 1793–1799
67. Lapham, C. K., Zaitseva, M. B., Lee, S., Romanstseva, T., and Golding, H. (1999) Nat. Med. 5, 303–308
68. Genoud, S., Kajumo, F., Guo, Y., Thompson, D., and Dragic, T. (1997) J. Virol. 73, 1645–1648
69. Blanpain, C., Lee, B., Vakili, J., Doranz, B. J., Govaerts, C., Migeotte, I., Sharron, M., Dupriez, V., Vassart, G., Doms, R. W., and Parmentier, M. (1999) J. Biol. Chem. 274, 18902–18908
70. Schertler, G. F., and Hargrave, P. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11578–11582
71. Unger, V. M., and Scheirer, G. F. (1995) Biophys J. 68, 1776–1786
72. Unger, V. M., Hargrave, P. A., Baldwin, J. M., and Schertler, G. F. (1997) Nature 399, 203–206