15. Thylakoids were purified from seedlings 10 to 14 days old. Approximately seven seedlings were pooled for each methylamine assay. Seedling leaf tissue was cut and then homogenized in grinding buffer as in (*9*). The homogenate was filtered through Miracloth, and chloroplasts were pelleted. The pellet was resuspended in lysis buffer [10 mM Hepes-KOH (pH 8.0) and 5 mM $MgCl_2$] and repelleted. Thylakoids were resuspended to chlorophyll (0.8 mg/ml) for the wild type and to an equivalent volume based on fresh mass for mutants. Mutant thylakoids were confirmed for phenotype by protein gel blots with antibodies against HCF106 (for *hcf106* mutants) or OE23 and PC (for *tha1* mutants). Methylamine assays were conducted in lysis buffer with 3 mM Mg-ATP, 5 mM dithiothreitol, $^{14}CH_3NH_2$ (1.67 μCi/ml), and thylakoids (chlorophyll, 0.2 mg/ml). Assays were conducted at 25°C in the presence of light. Thylakoids were collected on a 0.45-μm Millipore filter at 0, 2, and 5 min (~60 μg of chlorophyll per time point) and washed with lysis buffer. Methylamine accumulation was measured by scintillation spectroscopy. Unenergized thylakoids, generated by the addition of nigericin (0.75 μM final concentration) and valinomycin (1.5 μM final concentration), were used as a measure of background accumulation [H. Rottenberg, *Methods Enzymol.* **55**, 547 (1979)]. Average accumulations at 2 min in counts per minute ± SEM were as follows: wild-type, 9559 ± 1398; wild type with ionophores, 623 ± 106; *hcf106*, 2900 ± 961; *hcf106* with ionophores, 697 ± 260; *tha1*, 2528 ± 259; and *tha1* with ionophores, 616 ± 177.
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21. The HCF106 polyclonal antibodies were raised against a full-length TrpE::HCF106 fusion protein by standard methods. The antibodies were affinity-purified with the use of a MalE::HCF106 protein. Both unpurified and purified sera immunoprecipitate a 35-kD protein in wheat germ extract translations programmed with in vitro–transcribed Hcf106 cDNA. Protein immunoblot analysis detects a 30-kD HCF106 protein from maize leaf extracts. The 30-kD protein copurifies with chloroplasts and is protected by intact chloroplasts from thermolysin digestion. We have further confirmed that HCF106 is a chloroplast protein through import of in vitro–translated HCF106 into purified pea and maize chloroplasts (K. Cline and A. M. Settles, unpublished data). Both precursor and mature proteins migrate on SDS gels much more slowly than predicted by the deduced full-length protein sequence (27 kD).
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31. Import assays were conducted as in (*9*). Chloroplasts were purified from maize seedlings 10 to 12 days old, grown during 16-hour days at 26°C and 8-hour nights at 12°C. *hcf106* mutants were selected by their pale green phenotype, the aerial portions of the seedlings were harvested, and chloroplasts were purified with continuous Percoll gradients. Mutant chloroplasts were confirmed by protein immunoblot. Equal numbers of whole wild-type and *hcf106-mum3* mutant chloroplasts were incubated with radiolabeled maize precursors for OE33 (*13*) and OE17 [W. F. Ettinger and S. M. Theg, *Plant Physiol.* **99**, 791 (1992)] for 10 min at 25°C with light and Mg-ATP (5 mM). Chloroplasts were incubated with or without nigericin (0.75 μM), valinomycin (1.5 μM), and NaN_3 (10 mM) on ice 10 min before the addition of diluted precursor. The chloroplasts were post-treated with thermolysin (0.1 mg/ml) on ice for 40 min and repurified on a 35% Percoll cushion with 5 mM EDTA. The radiolabeled proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and visualized with fluorography.
32. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
33. Chloroplasts were prepared as described (*30*). Pelleted chloroplasts were hypotonically lysed by resuspension in 10 mM Hepes-KOH (pH 8.0) and 5 mM $MgCl_2$ and incubation on ice for 10 min. Thylakoids were purified by slow-speed centrifugation and washed once in lysis buffer. Supernatant proteins were concentrated by trichloracetic acid (TCA) precipitation. Membrane and supernatant pellets were resuspended in equal volumes of loading buffer, separated by SDS-PAGE, and blotted to nitrocellulose. The blot was incubated and stripped, and reprobed with several antibodies with the use of enhanced chemiluminescence.
34. Thylakoid membranes were prepared as described (*15*). Thylakoids were pelleted and resuspended (in 50 μg of chlorophyll per milliliter) in each chaotropic solution with 1 mM phenylmethylsulfonyl fluoride. The membranes were then incubated on ice for 30 min. Sonicated membranes were treated in a bath sonicator four times for 10 s each time. The extracted thylakoid membranes were separated by differential centrifugation. Supernatants were concentrated by TCA precipitation. The samples were separated by SDS-PAGE, and protein gels were blotted with antibodies against HCF106, OE33 (*13*), and LHCP.
35. Purified chloroplasts were lysed, and thylakoids were separated by low-speed centrifugation. The thylakoids were then resuspended in lysis buffer or 1% Triton X-100 (0.3 mg of chlorophyll per milliliter) and treated with thermolysin (0.1 mg/ml final concentration) for 30 min on ice. Protease digestion was stopped by addition of EGTA (50 mM final concentration), the samples were separated by SDS-PAGE, and protein gels were blotted with antibodies against HCF106 and OE33 (*13*).
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CD4-Independent Binding of SIV gp120 to Rhesus CCR5

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CCR5 and CD4 are coreceptors for immunodeficiency virus entry into target cells. The gp120 envelope glycoprotein from human immunodeficiency virus strain HIV-1(YU2) bound human CCR5 ($CCR5_{hu}$) or rhesus macaque CCR5 ($CCR5_{rh}$) only in the presence of CD4. The gp120 from simian immunodeficiency virus strain SIVmac239 bound $CCR5_{rh}$ without CD4, but $CCR5_{hu}$ remained CD4-dependent. The CD4-independent binding of SIVmac239 gp120 depended on a single amino acid, Asp^{13}, in the $CCR5_{rh}$ amino-terminus. Thus, CCR5-binding moieties on the immunodeficiency virus envelope glycoprotein can be generated by interaction with CD4 or by direct interaction with the CCR5 amino-terminus. These results may have implications for the evolution of receptor use among lentiviruses as well as utility in the development of effective intervention.

HIV-1 entry is mediated by the viral envelope glycoprotein complex consisting of the exterior glycoprotein, gp120, and the transmembrane glycoprotein, gp41 (*1*). CD4 acts as the primary target cell receptor, binding to the gp120 glycoprotein (*2*). The gp120-CD4 complex requires an additional target cell coreceptor of the seven-transmembrane-spanning G protein–coupled receptor family (*3–5*). Formation of this complex is believed to induce a conformational change exposing the gp41 ectodomain, which mediates fusion of the viral and target cell membranes (*6*).

Binding of CD4 to HIV-1 gp120 exposes structural epitopes detectable with monoclonal antibodies (mAbs) (*7*). These epitopes include conserved and variable structures on gp120 that are implicated in binding to the coreceptor (*4*, *5*) and suggest a stepwise mechanism by which macrophage-tropic strains of HIV-1 achieve fusion and entry into host cells. Sequential binding of CD4 followed by coreceptor may enable the virus to shield its fusion-sensitive epitopes from the immune system until contact with the target cell surface.

CCR5 cloned from rhesus macaques ($CCR5_{rh}$) is highly similar to human CCR5 ($CCR5_{hu}$) (98.3% sequence identity), with only eight amino acid changes (*8*). Five are conservative substitutions in transmembrane domains; the others are a $Lys^{171} \rightarrow$ Arg substitution in the second extracellular loop and two nonconservative substitutions in the NH_2-terminus (rhesus $\rightarrow$ human $Thr^9 \rightarrow$ Ile, $Asp^{13} \rightarrow$ Asn) (*8*). These substitutions do not restrict viral entry, as primary isolates of SIV and HIV efficiently infect $CD4^+$ cells expressing rhesus or human CCR5 (*8*, *9*). Rhesus and human CCR5 also support entry by interaction with a wide spectrum of SIV (macrophage- and T cell–tropic) and HIV (macrophage-tropic) envelope glycoproteins expressed on recombinant virions (*8*, *9*). The natural ligands for CCR5, the β chemokines MIP-1α, MIP-1β, and RANTES, inhibit infection by HIV-1, HIV-2, and SIV (*10*). Here, we examined the interactions of HIV-1 and SIV envelope glycoproteins with rhesus and human CCR5.

The system used involved intact HEK293 cells transiently transfected with expression plasmids for $CCR5_{rh}$ or $CCR5_{hu}$ (*11*). ^{125}I-labeled MIP-1β bound $CCR5_{hu}$ (*12*) and $CCR5_{rh}$ with dissociation constants (K_d) of 0.4 and 1.2 nM, respectively, comparable to the K_d of 0.85 nM observed for endogenous receptors on human primary macrophage cultures (*13*). ^{125}I-labeled MIP-1α bound with slightly lower affinity to either receptor (K_d = 3.5 nM, $CCR5_{rh}$; K_d = 6.4 nM, $CCR5_{hu}$). Thus, the transfected cell system provides an adequate model of the native conformation of these receptors.

HIV-1(YU2) is a primary macrophage-tropic isolate that uses CCR5 as a coreceptor (*4*). Recombinant YU2 gp120 envelope glycoprotein bound $CCR5_{rh}$ in transfected HEK293 cells with a K_d of 5.2 nM in the presence of 100 nM soluble CD4 (sCD4) (*14*). In the absence of sCD4, specific binding was not observed (Fig. 1). YU2 gp120 competed with ^{125}I-labeled MIP-1β for binding to rhesus or human CCR5 in the presence of sCD4 (*4*, *13*). Thus, YU2 gp120 binds to $CCR5_{rh}$ with an affinity nearly identical to that observed for $CCR5_{hu}$. As for $CCR5_{hu}$ (*4*, *5*), CD4 is required for efficient interaction of YU2 gp120 with $CCR5_{rh}$.

Rhesus and human CCR5 efficiently support entry of SIVmac239 (*8*, *9*) and other SIV strains (*8*) in CD4-expressing cells. Direct interactions among SIVmac envelope glycoproteins, CD4, and CCR5 have not been demonstrated. In the presence of 100 nM sCD4, SIVmac239 gp120 competed with ^{125}I-labeled SIVmac239 gp120 (Fig. 2A) or ^{125}I-labeled MIP-1α (Fig. 2B) for binding to $CCR5_{rh}$, with a K_d of 8.19 ± 1.06 nM (n = 11) (*14*). Binding was dependent on the presence of the V3 loop, as a SIVmac239 ΔV3 gp120 mutant lacking this region failed to compete with ^{125}I-labeled SIVmac239 gp120 or ^{125}I-labeled MIP-1α at 1000-fold molar excess (Fig. 2B). In contrast to CD4-dependent binding of macrophage-tropic HIV-1 glycoproteins, SIVmac239 gp120 bound $CCR5_{rh}$ without sCD4, with a K_d of 14.36 ± 3.90 nM (n = 11) (Fig. 2A). The difference in affinities with or without sCD4 was not statistically significant (P = 0.12); however, in the presence of sCD4, 20 to 40% more gp120 was bound. SIVmac239 gp120 also bound $CCR5_{hu}$ in HEK293 cells in the presence of sCD4, with a K_d of 2.7 nM (Fig. 3C). However, binding to $CCR5_{hu}$ was CD4-dependent (Fig. 2C).

Amino acid sequences in several distinct regions of CCR5 have been shown to be essential for coreceptor function (*15*, *16*). Using alanine scanning mutagenesis of $CCR5_{hu}$, we recently identified a region that included the tyrosine-rich motif Tyr^{10}-Asp-Ile-Asn-Tyr-Tyr (*17*). In $CCR5_{rh}$ this sequence is Tyr^{10}-Asp-Ile-Asp-Tyr-Tyr, and we hypothesized that this substitution may correlate with the CD4 independence of SIVmac239 binding to $CCR5_{rh}$.

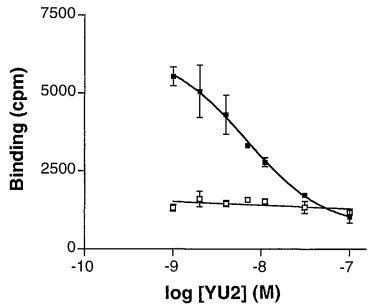

Fig. 1. HIV-1(YU2) gp120 binding to rhesus CCR5 in transiently transfected HEK293 cells. Cells (10^6) expressing $CCR5_{rh}$ were incubated with 1.0 nM ^{125}I-labeled YU2 gp120 in the presence (■) or absence (□) of 100 nM sCD4 and the indicated concentrations of unlabeled YU2 gp120. Samples were processed as described (*12*). Data are expressed as mean cpm ± SEM. Data are representative of six experiments.

To examine this possibility, we mutated $CCR5_{rh}$ from Asp^{13} to Asn^{13} as it occurs in $CCR5_{hu}$ (*18*). In transfected HEK293 cells, $CCR5_{rhD13N}$ bound SIVmac239 gp120 only in the presence of sCD4 (K_d = 11.6 nM) (Fig. 2D), which suggested that the single charge difference at residue 13 in the NH_2-terminus confers CD4-independent binding to rhesus but not human CCR5. To confirm this finding, we made the corresponding mutation ($Asn^{13} \rightarrow$ Asp) in $CCR5_{hu}$ (*18*). $CCR5_{huN13D}$ conferred gain of function for CD4-independent binding of SIVmac239 gp120 (K_d = 7.7 nM with sCD4, K_d = 0.85 nM without sCD4) (Fig. 2E). HIV-1(YU2) gp120 binding was CD4-dependent for both mutant receptors (*19*). The other NH_2-terminal mutation between $CCR5_{rh}$ and $CCR5_{hu}$ ($Thr^9 \rightarrow$ Ile) has no role in CD4 independence, as $CCR5_{rhT9I}$ retained CD4-independent binding of SIVmac239 gp120 (K_d = 11.4 nM with sCD4, K_d = 4.04 nM without sCD4) (*19*).

We tested seven mAbs derived from an HIV-2–infected human for their ability to inhibit SIVmac239 gp120 binding to $CCR5_{rh}$ in HEK293 cells in the presence and absence of sCD4. These mAbs recognize distinct conformation-dependent epitopes common to HIV-2 and multiple strains of SIV (*20*, *21*). The mAbs B23, 110C, or 17A, which react with SIVmac239 (*21*), efficiently inhibited CD4-independent binding of SIVmac239 gp120 (Fig. 3, A and B); mAb 15D, which has been reported to react with SIVmac251 and weakly with SIVmac239 (*20*), partially inhibited SIVmac239 gp120 binding (Fig. 3A). The mAbs 23F and 34G, which also do not react with SIVmac239, failed to inhibit SIVmac239 gp120 binding to $CCR5_{rh}$, and mAb 26C, a non-neutralizing mAb that reacts with SIVmac239, does not block CCR5 binding (Fig. 3A). Inhibition of SIVmac239 gp120

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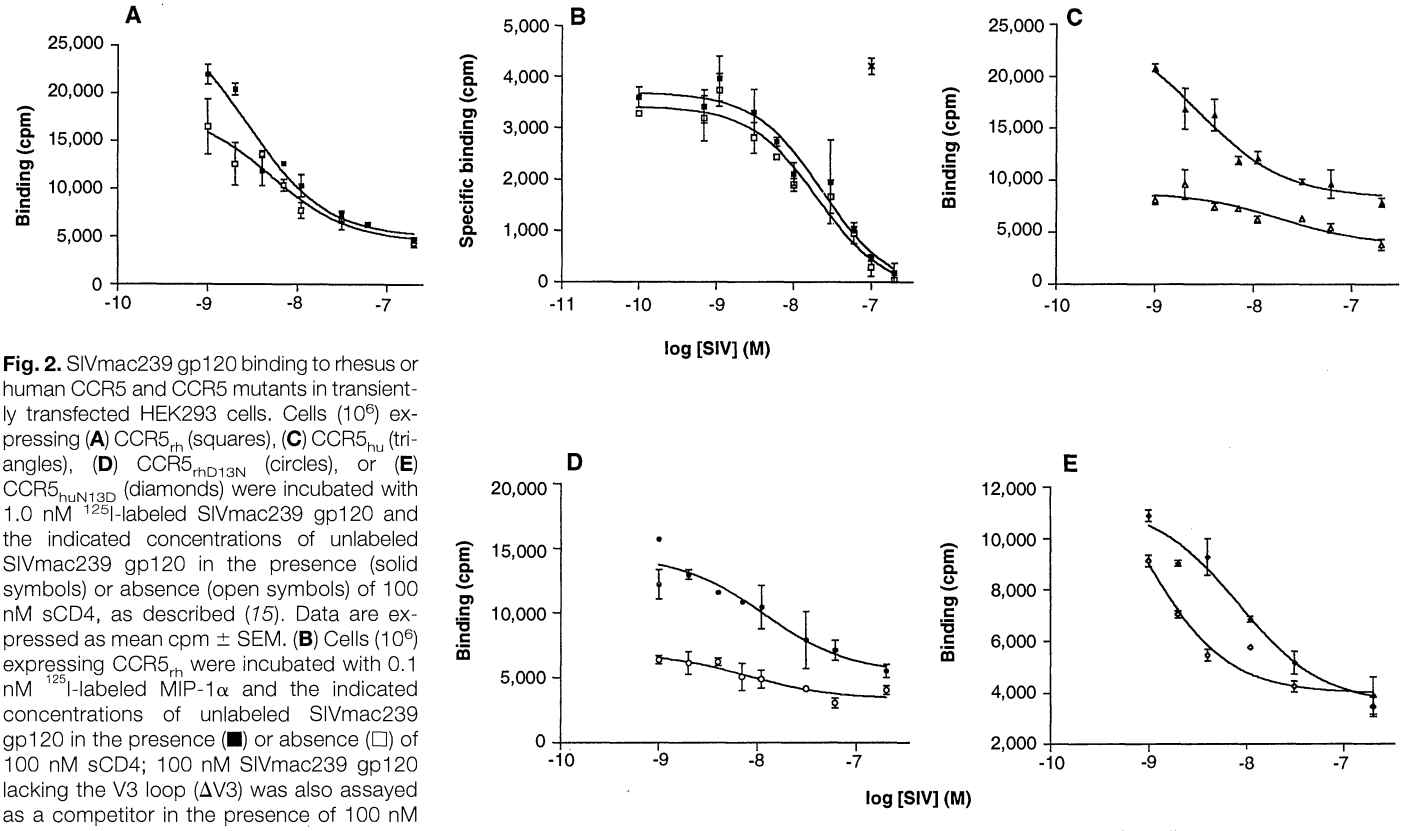

Fig. 2. SIVmac239 gp120 binding to rhesus or human CCR5 and CCR5 mutants in transiently transfected HEK293 cells. Cells (10^6) expressing (**A**) $CCR5_{rh}$ (squares), (**C**) $CCR5_{hu}$ (triangles), (**D**) $CCR5_{rhD13N}$ (circles), or (**E**) $CCR5_{huN13D}$ (diamonds) were incubated with 1.0 nM ^{125}I-labeled SIVmac239 gp120 and the indicated concentrations of unlabeled SIVmac239 gp120 in the presence (solid symbols) or absence (open symbols) of 100 nM sCD4, as described (*15*). Data are expressed as mean cpm ± SEM. (**B**) Cells (10^6) expressing $CCR5_{rh}$ were incubated with 0.1 nM ^{125}I-labeled MIP-1α and the indicated concentrations of unlabeled SIVmac239 gp120 in the presence (■) or absence (□) of 100 nM sCD4; 100 nM SIVmac239 gp120 lacking the V3 loop (ΔV3) was also assayed as a competitor in the presence of 100 nM sCD4 (X). Data are expressed as mean specific binding (cpm ± SEM), where nonspecific binding in the presence of 200 nM unlabeled SIVmac239 gp120 is subtracted from total binding. Data are representative of nine (A), two (B, C, and E), or three (D) independent experiments.

binding by neutralizing (but not non-neutralizing) mAbs suggests that these antibodies may interfere with the gp120-CCR5 interaction. Further, antibody inhibition in the absence of CD4 suggests they do not recognize CD4-induced epitopes.

The observation that some lentiviruses use chemokine receptors but not CD4 for entry raises the possibility that primordial lentiviruses used seven-transmembrane-spanning proteins as a sole receptor, and that the use of CD4 evolved with the primate lentiviruses (*22*). In this view, SIV represents a transitional virus between CD4-independent lentiviruses and HIV-1, for which CD4 use is obligate. This helps to explain the propensity of the related virus, HIV-2, to evolve virus strains that efficiently infect CD4-negative cells by use of chemokine receptors alone (*23*).

Infection by most SIV strains is significantly enhanced by the presence of CD4 on the target cells. The discrepancy between CD4-independent gp120 binding and CD4 enhancement of SIV infection may be explained by the different context in which these two processes occur. Entry is mediated by a trimeric gp120-gp41 complex on the viral surface (*24*), whereas the binding assays use gp120 monomers (*25*). CD4 binding may serve additional functions for intact virus besides the induction of the chemokine receptor binding site.

Conformation-dependent, CD4-independent neutralizing mAbs from HIV-2–infected individuals efficiently inhibited SIVmac239 binding to $CCR5_{rh}$ (Fig. 3, A and B). Alterations in the SIV gp120 V3 and V4 regions have been shown to disrupt a conformation-dependent epitope that is the target of the most potent anti-SIV neutralizing antibodies (*26*).

The aspartic acid residue that determines CD4-independent SIVgp120 binding resides within a tyrosine-rich motif identified by mutagenesis as critical for the interaction of CCR5 with the HIV-1 and SIV

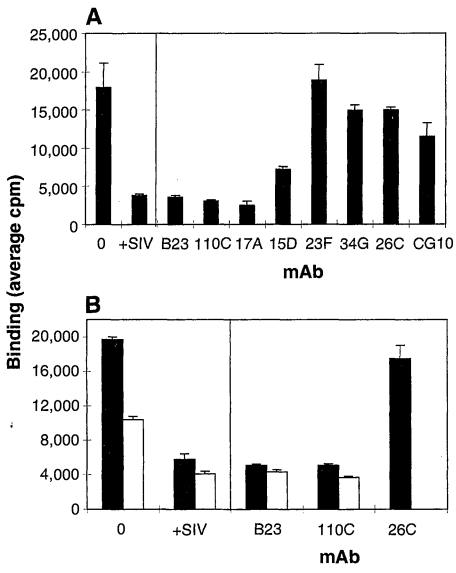

Fig. 3. HIV-2 mAb inhibition of ^{125}I-labeled SIVmac239 gp120 binding to rhesus CCR5 in transiently transfected HEK293 cells. (**A**) Cells (10^6) were incubated with 1.0 nM ^{125}I-labeled SIVmac239 gp120 and 100 nM sCD4 alone, with 300 nM unlabeled SIVmac239 gp120, or with 5 μg of the indicated HIV-2 mAbs [B23, 110C, and 17A react with SIVmac239 as well as other SIV strains (*20, 21*); 23F and 34G do not react with SIVmac239; 15D reacts weakly and 26G reacts strongly but is non-neutralizing; CG10 is specific for HIV-1 and was included as a negative control]. Data are expressed as mean cpm ± SEM and are representative of three independent experiments. (**B**) Antibody inhibition of ^{125}I-labeled SIVmac239 gp120 binding to rhesus CCR5 in the presence (solid bars) or absence (open bars) of 100 nM sCD4. (Competition by mAb 26C in the absence of CD4 was not tested.) Binding conditions are as described for (A). Data are means ± SEM and are representative of three independent experiments.

gp120 glycoproteins (*17*, *27*). This motif is conserved in all chemokine receptors used by primary HIV-1 and SIV isolates. Our results suggest that amino acid residues within this region directly contact the gp120 glycoprotein during CCR5 binding. It is likely that the interaction of CCR5 with a previously cryptic site on gp120 near or within the V3 loop triggers subsequent conformational changes in the envelope glycoprotein, leading to virus–cell membrane fusion. In HIV-1, CD4 binding is required to expose or form the CCR5 binding site. In SIV, gp120 moieties, perhaps within the V3 loop, can interact with Asp^{13} of $CCR5_{rh}$ and initiate high-affinity gp120-CCR5 binding in the absence of CD4. The identification of this single critical amino acid in CCR5 may have utility in the design of assay systems for therapeutics and vaccine candidates.

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