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A V3 Loop-Dependent gp120 Element Disrupted by CD4 Binding Stabilizes the Human Immunodeficiency Virus Envelope Glycoprotein Trimer

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Human immunodeficiency virus (HIV-1) entry into cells is mediated by a trimeric complex consisting of noncovalently associated gp120 (exterior) and gp41 (transmembrane) envelope glycoproteins. The binding of gp120 to receptors on the target cell alters the gp120-gp41 relationship and activates the membrane-fusing capacity of gp41. Interaction of gp120 with the primary receptor, CD4, results in the exposure of the gp120 third variable (V3) loop, which contributes to binding the CCR5 or CXCR4 chemokine receptors. We show here that insertions in the V3 stem or polar substitutions in a conserved hydrophobic patch near the V3 tip result in decreased gp120-gp41 association (in the unliganded state) and decreased chemokine receptor binding (in the CD4-bound state). Subunit association and syncytium-forming ability of the envelope glycoproteins from primary HIV-1 isolates were disrupted more by V3 changes than those of laboratory-adapted HIV-1 envelope glycoproteins. Changes in the gp120 β2, β19, β20, and β21 strands, which evidence suggests are proximal to the V3 loop in unliganded gp120, also resulted in decreased gp120-gp41 association. Thus, a gp120 element composed of the V3 loop and adjacent beta strands contributes to quaternary interactions that stabilize the unliganded trimer. CD4 binding dismantles this element, altering the gp120-gp41 relationship and rendering the hydrophobic patch in the V3 tip available for chemokine receptor binding.

The entry of human immunodeficiency virus type 1 (HIV-1) is mediated by the viral envelope glycoproteins (9, 79). The HIV-1 envelope glycoproteins are synthesized as an ~850-amino acid precursor, which trimerizes and is posttranslationally modified by carbohydrates to create a 160-kDa glycoprotein (gp160). The gp160 envelope glycoprotein precursor is proteolytically processed in the Golgi apparatus, resulting in a gp120 exterior envelope glycoprotein and a gp41 transmembrane envelope glycoprotein (16, 17, 66, 76). In the mature HIV-1 envelope glycoprotein trimer, the three gp120 subunits are noncovalently bound to three membrane-anchored gp41 subunits (32).

HIV-1 entry involves the binding of gp120 in a sequential fashion to CD4 and one of the chemokine receptors, CCR5 or CXCR4 (1, 8, 15, 18, 25, 36). CD4 binding triggers the formation of an activated intermediate that is competent for binding to CCR5 or CXCR4 (29, 69, 73, 78). These chemokine receptors are G protein-coupled, 7-transmembrane segment receptors with relatively short N termini. The choice of chemokine receptors is dictated primarily by the sequence of a gp120 region, the third variable (V3) loop, that exhibits variability among HIV-1 strains and becomes exposed upon CD4 binding (4, 8, 10, 33, 37, 38, 49, 59, 75). X-ray crystal structures of CD4-bound HIV-1 gp120 have revealed that the gp120 “core” consists of a gp41-interactive inner domain, a surface-exposed and heavily glycosylated outer domain, and a conformationally flexible bridging sheet (38, 43, 79). In the CD4-bound state, the V3 loop projects 30 Å from the gp120 core, toward the chemokine receptor (38). The V3 loop in these structures consists of three elements: (i) conserved antiparallel β strands that contain a disulfide bond at the base of the loop; (ii) a conformationally flexible stem; and (iii) a conserved tip (37, 38). During the virus entry process, the base of the gp120 V3 loop and elements of the bridging sheet interact with the CCR5 N terminus, which is acidic and contains sulfotyrosine residues (12–14, 23, 24). Sulfotyrosine 14 of CCR5 is thought to insert into a highly conserved pocket near the V3 base, driving further conformational rearrangements that result in the rigidification of the V3 stem (37). The conserved β-turn at the tip of the V3 loop, along with some residues in the V3 stem, is believed to bind the “body” of CCR5, i.e., the extracellular loops and membrane-spanning helices. CCR5 binding is thought to induce further conformational changes in the HIV-1 envelope glycoproteins, leading to the fusion of the viral and target cell membranes by the gp41 transmembrane envelope glycoproteins.

CCR5 binding involves two points of contact with the gp120 V3 loop: (i) the CCR5 N terminus with the V3 base and (ii) the CCR5 body with the V3 tip and distal stem (12–14, 23, 24, 37, 38). The intervening V3 stem can tolerate greater conforma-
HIV-1 envelope glycoproteins were added in triplicate to each well of a 96-well plate containing the target cells. The cocultivated cells were cultured at 37°C in a CO₂ incubator for 4 h. The medium was then removed, and the cells were washed once with PBS and lysed in 20 µl of lysis buffer. The plate was covered with Parafilm, wrapped in plastic film and place at ~70°C for 30 min. The β-galactosidase activity was measured with a Galacto-Star β-galactosidase reporter gene assay system for mammalian cells (Tropix, Bedford, MA), using a Berthold Microplate Luminometer LB 96V (Promega).

The ability of the wild-type and mutant HIV-1 ADA envelope glycoproteins expressed transiently in 293T cells to form synctia with target C127-CD4 cells expressing CD4 and either the wild-type CCR5 protein or the CCR5-GG mutant was assessed by using the α-complementation assay described above. The 293T cells were transfected with 0.8 µg of the envelope glycoprotein expression plasmid, 0.8 µg of the plasmid expressing the α-fragment of β-galactosidase, and 0.2 µg of the Tat-expressing plasmid. The C127-CD4 cells in 100-mm dishes were transfected with 2.5 µg of the plasmid expressing either wild-type CCR5 or CCR5-GG and 2.5 µg of the plasmid expressing the ω-fragment of β-galactosidase. On the next day, the transfected C127-CD4 cells were harvested with 5 mM EDTA-PBS and resuspended in a 96-well plate (2 × 10⁴ cells/well). The similar surface expression level of the wild-type CCR5 and the CCR5-GG proteins was verified by fluorescence-activated cell sorting using phycoerythrin-conjugated 2D7 antibody (BD Pharmingen). The medium of the 293T cells was changed on the day after transfection. The following day, the 293T cells were harvested with 5 mM EDTA-PBS, diluted to 2 × 10⁴ cells/ml with DMEM, and then cocultivated with the C127-CD4 cells as described above. After 4 h, the cells were lysed and β-galactosidase activity measured, as described above.

In some cases, an additional assay was used to measure the synctium-forming ability of the HIV-1 envelope glycoprotein variants (Table 1). In this assay, cells coexpressing the HIV-1 envelope glycoproteins and Tat were cocultivated with TZM-bl cells. Cell-cell fusion was quantitated by measuring luciferase activity (A. Finzi et al., unpublished data).

**Infection by single-round luciferase-expressing HIV-1.** Recombinant luciferase-expressing HIV-1 viruses were produced by transfection of 293T cells with the pCMV-Gag-Pol packaging plasmid, the pHIV-luc vector and the pSVII-env plasmids (50). At 3 days after transfection, the cell supernatants were harvested. The amount of virus in the supernatants was assessed by measurement of reverse transcriptase (RT) (57).

For infection, C127-CD4/CCR5 or C127-CD4/CXCR4 cells were plated at a density of 6 × 10⁴ cells/well in a 96-well plate. The following day, the cells were incubated with 2,500 RT units of recombinant virus per well. Two days later, the cells were lysed, and the luciferase activity measured in the Berthold microplate luminometer LB 96V (Promega).

**Immunoprecipitation of HIV-1 envelope glycoproteins.** 293T cells were transfected with pSVII-env plasmids expressing the HIV-1 envelope glycoproteins. One day later, the cells were metabolically labeled for 16 h with [35S]-Protein Labeling Mix (Perkin-Elmer). The cell lysates (containing gp160 and gp120) and media (containing gp120) were used for immunoprecipitation. Briefly, 400 µl of clarified cell lysate or medium was incubated with 100 µl of 10% protein A-Sepharose beads (Amersham Biosciences) and 4 µl of a mixture of a sera from HIV-1-infected individuals. For some experiments, 1 µg of a monoclonal antibody was used instead of the serum mixture. The mixtures were brought to a volume of 1 ml with PBS and incubated on a shaking platform at room temperature for 1 h. The immunoprecipitation of HIV-1 gp120-containing cell supernatants by the G3-299 monoclonal antibody was carried out at 4°C for 2 h in the presence of protease inhibitors (one tablet Complete protease inhibitor cocktail [mini, EDTA-free] per 10-ml binding reaction; Roche Applied Science, Germany). The pelleted beads were washed once with 0.5 M NaCl-PBS and twice with PBS. The beads were then suspended in a 2× loading buffer, boiled, and applied to a 10% sodium dodecyl sulfate-polyacrylamide gel. The CCR5 binding assay. To assess CCR5-binding ability, normalized amounts of radiolabeled gp120 envelope glycoproteins from transfected 293T cell supernatants were incubated with sCD4 and C127-CCR5 cells. Briefly, C127-CCR5 cells were transfected from the plate by using 5 mM EDTA-PBS (pH 7.5). After a wash with DMEM, the cells were resuspended in DMEM and added to 1.5 ml microcentrifuge tubes (3 × 10⁴ cells/tube). The radiolabeled gp120-containing cell supernatants (500 µl) and 10 µg of CD4 were added to the tube, and the volume was adjusted to 1 ml with DMEM. The tubes were rocked at room temperature for 1 h. The cell lysates were precipitated with a mixture of sera from HIV-1-infected individuals and protein A-Sepharose beads (Amersham Biosciences) at 4°C for 2 h. The precipitated gp120 was analyzed by SDS-PAGE and autoradiography.
RESULTS

HIV-1 V3 loop insertion mutants. To examine the effects of changes in the length or orientation of the HIV-1 gp120 V3 loop, a panel of insertion mutants derived from the ADA HIV-1 isolate was created (Fig. 1). The ADA virus is a primary, CCR5-using (R5) HIV-1 isolate (27). In one set of mutants, one or two glycine residues were introduced into one strand of the conformationally flexible stem of the V3 loop; these asymmetric insertions would presumably kink the loop. In a second set of mutants, identical insertions were placed symmetrically into the N- and C-terminal strands of the V3 stem; the intent of these changes was to extend the length of the V3 loop while minimizing any alterations in the orientation of the tip.

The ability of the V3 insertion mutants to mediate the fusion of envelope glycoprotein-expressing cells and cells bearing CD4 and CCR5 was assessed. Plasmid DNAs expressing the mutant envelope glycoproteins and the /H9251 fragment of /H9252-galactosidase were transfected into 293T cells. The 293T cells were cocultivated with canine Cf2Th cells expressing human CD4, human CCR5 and the /H9275 fragment of /H9252-galactosidase. Successful fusion of the Env-expressing cells with the Cf2Th-CD4/CCR5 cells results in reconstitution of enzymatically active /H9252-galactosidase (35). In this cell-cell fusion assay, most of the ADA envelope glycoproteins. These four mutants contain glycine insertions in the C-terminal strand of the V3 stem.

To examine the generality of these results, V3 mutants 1 and 2 were created in the context of envelope glycoproteins derived from two additional primary viruses, the R5 YU2 strain and the dualtropic (R5X4) 89.6 strain (11, 47), and from two CXCR4-using (X4) laboratory-adapted viruses, HXBc2 and MN27 (26, 64) (Fig. 1). The syncytium-forming abilities of these envelope glycoproteins were assessed as described above. For the envelope glycoproteins from the HXBc2 and MN27 HIV-1 strains, the mutant 1 glycoproteins, which contain a single glycine inserted into the N-terminal strand of the V3 stem, exhibited 80 to 85% of the syncytium-forming abilities of the respective wild-type envelope glycoproteins (Fig. 2B). In the context of the YU2 and 89.6 envelope glycoproteins, the mutant 1 insertion more significantly reduced syncytium-forming ability. Likewise, the HXBc2 and MN27 mutant 2 envelope glycoproteins, which contain two glycine residues inserted into the N-terminal strand of the V3 stem, exhibited 50 and 70% of the syncytium-forming abilities of the respective wild-type envelope glycoproteins. In contrast, the mutant 2 variants of the YU2 and 89.6 envelope glycoproteins induced only very low numbers of syncytia. The dualtropic 89.6 envelope glycoprotein variants induced fusion with target cells expressing CD4 and CCR5 or CD4 and CXCR4 equivalently. Thus, insertions in the V3 stem generally reduced the ability of HIV-1 envelope glycoproteins to induce cell-cell fusion. The extent of this reduction depended upon the number of residues inserted...
and upon the particular HIV-1 envelope glycoproteins altered, with the laboratory-adapted HIV-1 envelope glycoproteins being functionally more tolerant of these changes than the primary HIV-1 envelope glycoproteins.

Infectivity of HIV-1 with V3 loop insertions. The ability of the V3 insertion mutants to support HIV-1 infection was assessed in a single-round Env complementation assay (31). Regardless of the HIV-1 strain from which the envelope glycoproteins were derived, all of the V3 mutants were markedly defective in mediating HIV-1 entry (Fig. 2C and D). Complete defectiveness was also observed for an ADA envelope glycoprotein mutant with an alanine substitution in place of the glycine insert in mutant 1 (data not shown).

Expression, processing, and subunit association of the HIV-1 Env mutants. To investigate the basis for the reduced activities of the V3 mutants in syncytium-forming ability and virus replication, cells transiently expressing the wild-type and mutant envelope glycoproteins were radiolabeled. Cell lysates and supernatants were precipitated by a polyclonal mixture of sera from HIV-1-infected individuals. In the cell lysates, the wild-type gp160 envelope glycoprotein precursor and the mature gp120 envelope glycoprotein were evident (Fig. 3A and B). For the primary HIV-1 ADA, YU2, and 89.6 envelope glycoproteins, although the levels of the V3 mutant gp160 envelope glycoproteins were generally similar to those of the wild-type counterparts, the levels of cell-associated gp120 glycoproteins were relatively reduced (Fig. 3A and B). For these HIV-1 envelope glycoproteins, the amounts of gp120 shed into the medium were increased for the V3 mutants compared to the wild-type glycoproteins. These results suggest that the V3 loop insertions decrease the association of the gp120 and gp41 subunits in the unliganded envelope glycoprotein complex. The amount of cell-associated gp120 glycoprotein was lower for the wild-type HXBc2 and MN27 envelope glycoproteins than for the primary HIV-1 envelope glycoproteins, under these labeling conditions (Fig. 3B). Slight decreases in the amount of cell-associated gp120 were observed for the HXBc2 m1 and m2 mutants, relative to the wild-type HXBc2 envelope glycoproteins; however, no differences between the phenotypes of the MN27 wild-type and mutant envelope glycoproteins with respect to gp160 precursor processing or gp120-gp41 association were evident. Thus, the V3 loop insertions decrease gp120-gp41 association of primary HIV-1 envelope glyco-
proteins; this phenotype is less evident for envelope glycoproteins derived from laboratory-adapted HIV-1 isolates.

Decreased stability of the envelope glycoprotein complex explains the lower function of the V3 loop mutants in mediating virus entry compared to cell-cell fusion. Because a greater time interval elapses between envelope glycoprotein synthesis and engagement of the target cell in the virus entry assay, decreases in the functional stability of the HIV-1 envelope glycoproteins are more apparent than in the syncytium formation assay (84; Finzi et al., unpublished).

Interaction of mutant envelope glycoproteins with ligands.

To assess the effect of the V3 loop insertions on gp120 conformation, the recognition of the gp120 glycoprotein by conformation-dependent ligands was assessed. Radiolabeled gp120 glycoproteins from transfected cell supernatants were precipitated by a mixture of sera from HIV-1-infected individuals, which recognizes gp120 independently of its conformational integrity, or by specific gp120-directed ligands whose recognition depends upon gp120 conformation. The ligands include: CD4-Ig, in which the amino-terminal two domains of CD4 are fused to an immunoglobulin Fc; 17b and 412d, two antibodies that recognize CD4-induced epitopes near the chemokine receptor-binding surface of gp120 (58, 72); and 39F, which recognizes a conformation-dependent V3 epitope (45). All of the HIV-1 ADA gp120 mutants tested bound CD4 efficiently, as evidenced by the precipitation of these glycoproteins by CD4-Ig (Fig. 4A). The 17b and 412d antibodies precipitated all of the gp120 mutants, although the recognition of some of the mutants by the 17b antibody was less efficient than that of the wild-type gp120 glycoprotein. This result is consistent with the proximity of the 17b epitope to the base of the V3 loop (43, 58). The 39F anti-V3 antibody precipitated most of the mutants equivalently to the wild-type gp120, with three exceptions. Mutants 4, 6, and 9 were precipitated less efficiently than the wild-type gp120 glycoprotein by the 39F antibody. All three mutants have a two-residue insertion in the carboxy-terminal half of the V3 stem, suggesting that changes in this V3 region can disrupt the 39F epitope. These results suggest that the gp120 V3 insertion mutants are not globally misfolded but exhibit specific alterations near or within the V3 loop.

The ability of the gp120 mutants to bind CCR5 was examined (78). Radiolabeled gp120 glycoproteins from transfected cell supernatants were incubated in the presence of soluble CD4 with Cf2Th cells expressing CCR5. After a washing step, the amount of gp120 bound to the cells was determined. Figure 4B shows that all of the V3 insertions decreased the efficiency of CCR5 binding. This decrease was readily apparent for the gp120 glycoproteins from the R5 HIV-1 strains, ADA and YU2. The wild-type gp120 glycoprotein from the R5X4 (dual-tropic) 89.6 HIV-1 strain exhibits a lower affinity for CCR5 than those of R5 gp120 glycoproteins (2, 3). Therefore, higher concentrations of the 89.6 gp120 are required to demonstrate CCR5 binding. At these higher concentrations, mutants 1 and 2 from the 89.6 strain bound CCR5 less efficiently than the wild-type 89.6 gp120 glycoprotein (Fig. 4B). Thus, insertions into the V3 stem of the HIV-1 gp120 glycoprotein result in significant decreases in the efficiency of CCR5 binding.

Partial compensation of the syncytium-forming ability of V3 insertion mutants by a CCR5 protein with an extended amino terminus. Current models of HIV-1 gp120-CCR5 interaction suggest that the CCR5 N terminus binds near the V3 base and the body of CCR5 binds the V3 tip (12–14, 23, 24, 37, 38). Thus, the gp120 mutants with insertions in the V3 stem might utilize CCR5 less efficiently because of poor alignment of these two binding contacts. In this case, the mutants might be more effective if the CCR5 N terminus were extended further from the body of the chemokine receptor. To test this, two glycine
residues were inserted into the CCR5 sequence, between the N terminus and the initial cysteine residue. This mutant, CCR5-GG, and wild-type CCR5 were expressed in C12Th-CD4 cells, which were assessed for the ability to form syncytia with cells expressing the wild-type or mutant ADA envelope glycoproteins. The syncytium-forming ability of some of the V3 mutant envelope glycoproteins, relative to that of the wild-type ADA envelope glycoproteins, was greater with target cells expressing the CCR5-GG mutant than with target cells expressing wild-type CCR5 (Fig. 5). This phenotype was particularly evident for envelope glycoprotein mutants (mutants 7, 8, and 9) with symmetrical substitutions in the V3 loop and was also seen for mutant 1 with a single glycine insertion. The defective entry of viruses with the mutant envelope glycoproteins was not compensated by the expression of the CCR5-GG mutant on the target cell (data not shown). These results are consistent with a model suggesting that one of the consequences of the V3 insertions is a disruption of the optimal spacing between gp120 regions important for binding the CCR5 N terminus and body.

**A conserved V3 element that is important for gp120-gp41 association.** The effect of V3 loop insertions on gp120-gp41 association could result from an incompatibility of a kinked or extended loop with the proper packing of the gp120 subunits in the trimeric spike. Alternatively, specific structures in the V3 loop could positively contribute to subunit association. Although the V3 region is not absolutely essential for gp120-gp41 association in the laboratory-adapted HXBc2 envelope glycoproteins (see Fig. 3C), mild increases in the amount of gp120 spontaneously shed into the medium have been observed for V3 loop-deleted envelope glycoproteins compared to the wild-type HXBc2 envelope glycoproteins (81). To examine the role of the V3 loop in maintaining the integrity of the trimeric envelope glycoprotein complex in a primary HIV-1 isolate, the V3 loop of the ADA gp120 glycoprotein was deleted in precisely the same manner as that used previously for the HXBc2 envelope glycoproteins from a laboratory-adapted HIV-1. The V3 deletion resulted in a decrease in gp120-gp41 association comparable to that which resulted from an insertion into the V3 region (see Fig. 3D). Thus, the absence of the V3 loop can weaken gp120-gp41 interactions in the envelope glycoprotein trimer of a primary HIV-1 isolate.

The result described above raised the possibility that a V3 loop element contributes in a positive way to subunit association. Our observation that V3 insertions decreased the stability of the envelope glycoprotein trimers from multiple primary HIV-1 strains suggested that such a V3 element might be conserved in different HIV-1 isolates. Thus, we shifted our attention away from the more variable V3 stem to the relatively conserved V3 tip. Although the structure of the V3 loop in the unliganded HIV-1 envelope glycoproteins is unknown, the CD4-bound gp120 is known, in the CD4-bound gp120 (38), a β-hairpin at the V3 tip juxtaposes residues 307, 309, and 317 (Fig. 6, column at far right). Some variability is tolerated in these residues in HIV-1 strains; however, the hydrophobic character of these residues is almost always maintained (Fig. 7A). We hypothesized that this hydrophobic patch in the V3 tip serves to strengthen gp120-gp41 association in the unliganded HIV-1 envelope glycoprotein trimer. Amino acid residues of different degrees of hydrophobicity were introduced into residues 307, 309, and 317 of the HIV-1 YU2 envelope glycoproteins. Although hydrophobic substitutions did not significantly disrupt gp120-gp41 association, substitutions of alanine or hydrophilic residues resulted in severe decreases in subunit association (Fig. 7B to D). Thus, the hydrophobic patch in the V3 tip contributes to subunit association in the unliganded HIV-1 envelope glycoprotein trimer.

**Contribution of the V3 hydrophobic patch to HIV-1 envelope glycoprotein function.** The V3 tip is known to contribute to CCR5 binding (8, 9, 12, 14, 34, 58). The effect of changes in the hydrophobic V3 patch on CCR5 binding and syncytium formation were examined. Decreases in CCR5-binding affinity resulted from hydrophilic substitutions in residues 307, 309, and 317 (Fig. 4C and Table 1). These decreases were accompanied by defects in the ability to mediate the formation of syncytia (Table 1). The effects of changes in the hydrophobic V3 patch were in some cases more disruptive of gp120-gp41 association than of CCR5 binding and syncytium formation; in
general, however, the phenotypic effects of the V3 changes on all of these properties correlated.

Involvement of V3-proximal gp120 regions in association with gp41. Previous studies have suggested that, in the unliganded gp120 glycoprotein, the V3 loop may interact with other gp120 regions, particularly the β19, β20, and β21 strands (the fourth conserved [C4] region) and the V1/V2 stem-loop structure (51–53, 67, 74, 80). Several residues conserved among the gp120 glycoproteins of different HIV-1 strains were identified in these regions. The effect of alteration of these residues in the HIV-1 envelope glycoproteins on the association of gp120 with gp41 was examined (Table 1). Some changes in the β19, β20, and β21 strands resulted in decreased subunit association (Table 1 and Fig. 7D). In addition, changes in leucine 120 and valine 122 in the β2 strand of the conserved V1/V2 stem also reduced gp120-gp41 association. Thus, changes in several HIV-1 gp120 regions that may be proximal to the V3 loop in the unliganded state resulted in decreases in the association of gp120 with gp41.

Previous studies have examined the effect of the HIV-1 gp120 changes on gp120-gp41 association (32, 63, 71, 84; Finzi et al., unpublished). The results of these studies, as well as the results generated in the present study, are summarized in Fig. 6. The N and C termini and inner domain of gp120, particularly the inner domain β-sandwich, are the major contributors to the noncovalent interaction with gp41 (43, 55, 63, 84; Finzi et al., unpublished). Our results indicate that other gp120 regions (the V3 loop, β2, β17, β19, β20, and β21) can also influence the stability of the association of gp120 and gp41. A comparison of the different crystallized gp120 structures indicates that these regions can change conformation in response to ligand binding or alterations introduced into gp120 to promote crystallization. Although the structure of the unliganded HIV-1 gp120 glycoprotein is unknown, in light of this conformational flexibility, these gp120 elements are potentially in proximity to the unliganded envelope glycoprotein trimer.

Effects of gp120 changes on a discontinuous V3-C4 epitope. The G3-299 monoclonal antibody has been previously shown to recognize a discontinuous HIV-1 gp120 epitope that is apparently composed of elements from the V3 loop and the fourth conserved (C4) region (51, 52). The G3-299 antibody neutralizes laboratory-adapted HIV-1 strains (60,
indicating that this epitope is both intact and exposed on the unliganded envelope glycoprotein trimers of at least some HIV-1 variants. The effect of gp120 changes on the integrity of this epitope was examined by precipitation of a large panel of wild-type and mutant gp120 envelope glycoproteins by the G3-299 antibody. Of note, changes in the V3 loop (residues 307, 309, and 317) or in the H21 strand (residues 434 and 435) significantly decreased HIV-1 gp120 recognition by the G3-299 antibody (Tables 1 and 2). In the CD4-bound state, the only gp120 domains are colored as follows: outer domain (yellow), inner domain (red) and bridging sheet components (blue for the β20-β21 loop and green for the β2-β3 V1/V2 stem). In the cases where the V3 loop structure was not determined, the position of the V3 base is indicated. In the CD4-bound structure, the elements of the V3 loop are labeled. The gp120 beta strands (defined in the CD4-bound structure [43]) relevant to the present study are also labeled. In the bottom row, the gp120 residues are colored according to the gp120-gp41 association index (red, association index < 0.5; green, association index ≥ 0.5) observed upon mutagenesis of the HIV-1 YU2 and HXBc2 gp120 glycoproteins (32, 63, 71, 84; the present study; Finzi et al., unpublished). In the CD4-bound gp120 structure, the three hydrophobic residues in the tip of the V3 loop that were implicated in gp120-gp41 association are labeled.

Substitution of a tryptophan residue for serine 375 resulted in a decrease in the recognition of gp120 by the G3-299 anti-
body (see Table 1 footnotes). Because serine 375 contacts the F105 antibody, which does not compete with G3-299 for gp120 binding (6), the effects of the S375W change on G3-299 binding are likely indirect. Indeed, it has been shown that the S375W mutant favors the CD4-bound conformation (83), which is recognized less efficiently by the G3-299 antibody (52).

The V3 loop and adjacent gp120 elements in the unliganded HIV-1 envelope glycoprotein trimer. The X-ray crystal structure
of the CD4-bound HIV-1 gp120 envelope glycoprotein with an intact V3 loop (38) has been fitted to tomograms derived from cryo-electron microscopy studies of HIV-1 virion spikes (48). In the CD4-bound state, the exposed V3 loop projects obliquely from the outer domain toward both the trimer axis and the target cell (Fig. 6). Although the detailed structure of unliganded HIV-1 gp120 with an intact V3 loop is unknown, low-resolution electron cryotomograms of the unliganded HIV-1 gp120 envelope spike are available (48, 88). The unliganded SIV gp120 core structure (5) cannot be accommodated within the electron density of the unliganded HIV-1 envelope glycoprotein spike (48). Thus, the structure observed in the crystallized SIV gp120 core is distinct from that in the unliganded HIV-1 envelope glycoprotein complex, due to real or artifactual differences between the unliganded SIV and HIV-1 gp120 glycoproteins. Both the CD4-bound and the b12 antibody-bound HIV-1 gp120 core crystal structures can be readily fitted to the electron cryotomograms of the unliganded HIV-1 envelope glycoprotein spike (6, 48). The position and orientation of the V3 base in these models and the deduced proximity of the V3 loop to the β21 strand in the unliganded HIV-1 envelope glycoproteins (see above) suggests that the V3 loops likely project toward the trimer axis (Fig. 9 and data not shown). Thus, in the unliganded HIV-1 envelope glycoproteins, the three V3 loops are potentially poised for interactions with gp41 and/or the other gp120 elements implicated in stabilizing the association with gp41.

**DISCUSSION**

Here we show that the insertion of amino acid residues into the stem of the V3 variable loop of the gp120 envelope glyco-
TABLE 2. Ligand binding by HIV-1 gp120 variants

<table>
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<th>Binding</th>
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The wtA protein and the wtA mutants in the table are derived from the HIV-1 Yu2gp120 glycoprotein. Relative to gp120, the wtA protein and the mutant wtA proteins have a Δ282 N-terminal deletion and a Δ128-194 deletion of the V1/V2 variable loops (58).

The relative values for CD4 and CCR5 binding were taken from Rizzuto et al. (58). ND, not determined.

Radiolabeled wild-type and mutant gp120 glycoproteins in the supernatants were precipitated with the G3-299 monoclonal antibody, which recognizes the unliganded gp120 monomer better than the CD4-bound gp120 (51, 55). These models are consistent with the expected placement of conserved and variable gp120 surfaces, glycosylation sites, epitopes for neutralizing and non-neutralizing antibodies and receptor-binding regions (44). In these models, the V3 loop projects from the gp120 outer domain toward the trimer axis. Fixation of the assembled HIV-1 envelope glycoproteins, but not the gp120 monomer, by treatment with chemical crosslinkers has been shown to decrease specifically the binding of antibodies directed against the V3 loop and the gp41-interactive face of gp120 (85). These observations are consistent with the close packing of the V3 loops of the gp120 subunits near the trimeric axis of the unliganded HIV-1 envelope glycoprotein complex. This arrangement may help in sequestering the CCR5-binding elements of the V3 loop away from potential recognition by neutralizing antibodies.

The requirement to avoid the binding of potentially neutralizing antibodies constrains the structure of the unliganded envelope glycoproteins from primary HIV-1 isolates. Such constraints are removed by extensive passage of HIV-1 in tissue-cultured cell lines (56, 61, 68, 77). Short of destabilizing gp120-gp41 association, V3 variation may modulate quaternary subunit interactions that determine sensitivity to neutralizing antibodies. Indeed, in one case, a dualtropic HIV-1 retained some function after truncation of the V3 loop but was more sensitive to neutralization by antibodies (46). Likewise, changes in V3 can determine differences in sensitivity to neutralizing antibodies, even those directed against conserved gp120 epitopes, between primary and laboratory-adapted HIV-1 isolates (67, 86). V3 changes have also been shown to account for the increased neutralization resistance of simian-human immunodeficiency viruses passaged in monkeys (7, 21). Thus, alterations in the gp120 V3 loop can modulate sensitivity to neutralization by antibodies directed against multiple envelope glycoprotein determinants.

The syncytium-inducing function of the envelope glycoproteins was disrupted to a greater extent by V3 loop insertions for primary HIV-1 isolates compared to laboratory-adapted viruses. Deletion of the V3 loop exerted an effect on gp120-gp41 association that was greater for the primary HIV-1 ADA envelope glycoproteins than for the laboratory-adapted HXBc2 envelope glycoproteins. Several studies have suggested that the V3 loops of laboratory-adapted HIV-1 envelope glycoproteins are more exposed than the V3 loops of primary virus envelope glycoproteins; in some cases, these differences were apparent even on the monomeric gp120 glycoproteins (4, 20, 28, 46, 49, 65, 85). The decreased accessibility of the primary HIV-1 V3 loops may be a consequence of intramolecular contacts with other gp120 elements that ultimately modulate quaternary interactions on the trimer. Based on the phenotypic effects of gp120 amino acid changes on gp120-gp41 association, candidates for these gp120 elements include the V1/V2 stem (β2), B20-B21, and β17-β19. Perhaps, in the unliganded trimer, structures formed by the interaction of V3 with these elements help to create a stable binding interface with gp41, either by direct contact or indirectly. The proximity of V3 and β21 is supported by the inclusion of these regions in the discontinuous epitope for the G3-299 monoclonal antibody, which recognizes the unliganded HIV-1 gp120 monomer better than the CD4-bound gp120 (51, 52). Changes in gp120 residues 307, 309, and 317 in the hydro-
phobic patch in the V3 tip and in methionine 434 in β21 specifically disrupted the G3-299 epitope. A poorly replicating simian immunodeficiency virus altered in a single residue (equivalent to methionine 434 in HIV-1) reverted by changing the equivalent of V3 residue 307, further suggesting that the V3 hydrophobic patch may be proximal to β21 in the unliganded envelope glycoproteins (53). Changes in the V3 hydrophobic patch and a V1/V2 loop segment near the V1/V2 stem have recently been shown to disrupt the HIV-1 trimer-specific epitopes recognized by the broadly neutralizing PG9 and PG16 antibodies (74). This observation supports the proximity of the V3 tip and the V1/V2 stem and suggests that both of these elements reside close enough to the trimer axis to be influenced by quaternary interactions among the subunits of the unliganded HIV-1 envelope glycoprotein.

The multiple intramolecular contacts required to maintain trimer integrity may impose limitations on the tolerance of primary HIV-1 envelope glycoproteins to V3 loop insertions and deletions. Despite variation in particular amino acid residues, the length of the gp120 V3 loop is very well conserved among primary HIV-1 strains, with rare exceptions (42). For example, in the envelope glycoproteins of some group M HIV-1 isolates, one or two amino acid residues are inserted into the carboxy-terminal strand of the V3 loop, compared to the sequence of most HIV-1 strains. The site of these natural insertions in the V3 stem corresponds precisely to the insertion site in mutants 3 and 4. Interestingly, mutants 3 and 4 retained some function in the cell-cell fusion assay, in contrast to most of the other mutants. The V3 loops of the group O (outlier group) of HIV-1 are also longer compared to those of most group M viruses, again due to insertions in the carboxy-terminal strand of the loop (42). Thus, at least some length variation in the carboxy-terminal V3 stem can be functionally tolerated, although levels of cell-cell fusion induced by mutants with changes in this region were lower than that observed for the wild-type envelope glycoproteins. Naturally occurring HIV-1 strains with V3 insertions may have evolved compensatory changes in other parts of the envelope glycoproteins.

CD4 binding results in exposure of the V3 loop (49, 59), even in the context of monomeric gp120 (75). In a crystal structure of CD4-bound gp120 with an intact V3 region, the tip of the V3 loop is located ~30 Å away from the gp120 core (38) (Fig. 6 and 8). Thus, any potential interaction between the V3 tip/stem and the gp120 core in the unliganded conformation is disrupted upon CD4 binding. This is consistent with the observation that CD4 binding decreased the binding of gp120 by the G3-299 antibody, which recognizes a discontinuous epitope composed of V3 and β21 sequences (51, 52). Moreover, mutant HIV-1 gp120 glycoproteins (H66A and W69L) that spontaneously sample the CD4-bound conformation less than wild-type gp120 (39; Finzi et al., unpublished) were recognized more efficiently than the wild-type gp120 by G3-299 (Table 1 legend). Combined with a recent study on CD4-induced conformational changes in the topological layers (“layers 1 and 2”) of the gp120 inner domain (Finzi et al., unpublished), our observations allow a more comprehensive picture of the network of gp120 rearrangements that occur upon CD4 binding (Fig. 10).
CD4-induced conformational changes in the V3 loop might alter the gp120-gp41 interaction and help prime gp41 for subsequent steps in the membrane fusion process.

Current models of HIV-1 gp120-CCR5 binding propose two critical points of contact: (i) between the gp120 V3 base-bridging sheet and the CCR5 N terminus and (ii) between the V3 tip and the body of CCR5 (12–14, 23, 24, 37, 38). The hydrophobic patch in the V3 tip may interact with the hydrophobic pocket thought to be formed by the membrane-spanning helices of CCR5 (19, 40, 54, 62). Although the stem that separates the V3 base and tip can tolerate both sequence variation and conformational flexibility (38, 42), changes in length appear to be more disruptive of chemokine receptor binding. All of the V3 insertions studied herein resulted in significant decreases in CCR5 binding. Variation in the length or conformation of the V3 stem presumably interferes with the precise spatial relationship of the two CCR5-interactive moieties on gp120, precluding high-affinity binding. This interpretation is consistent with the ability of a mutant CCR5 receptor with an extended N terminus to compensate partially for some of the V3 loop insertions.

Future studies should allow a more detailed understanding of the structural relationships involving the V3 loop in the unliganded HIV-1 envelope glycoprotein trimer, and the contribution of the V3 loop to receptor-induced conformational transitions leading to HIV-1 entry.

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