JOURNAL OF VIROLOGY, June 1993, p. 3674–3679
0022-538X/93/063674-06$02.00/0

Vol. 67, No. 6

Effect of Amino Acid Changes in the V1/V2 Region of the Human Immunodeficiency Virus Type 1 gp120 Glycoprotein on Subunit Association, Syncytium Formation, and Recognition by a Neutralizing Antibody

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Received 5 November 1992/Accepted 9 March 1993

The contributions of the first and second variable regions of the human immunodeficiency virus type 1 gp120 glycoprotein to envelope glycoprotein structure, function, and recognition by a neutralizing antibody were studied. Several mutants with substitutions in the V2 loop demonstrated complete dissociation of the gp120 and gp41 glycoproteins, suggesting that inappropriate changes in V2 conformation can affect subunit assembly. Some glycoproteins with changes in V1 or V2 were efficiently expressed on the cell surface and were able to bind CD4 but were deficient in syncytium formation and/or virus entry. Recognition of gp120 by the neutralizing monoclonal antibody G3-4 was affected by particular substitutions affecting residues 176 to 184 in the V2 loop. These results suggest that the V1/V2 variable regions of the human immunodeficiency virus type 1 gp120 glycoprotein play a role in postreceptor binding events in the membrane fusion process and can act as a target for neutralizing antibodies.

Human immunodeficiency virus type 1 (HIV-1) is the etiologic agent of AIDS (2, 12). The initial interaction of HIV-1 with the target cell involves the binding of the viral exterior envelope glycoprotein, gp120, to the CD4 glycoprotein, which acts as the viral receptor (1, 7, 24, 31, 32, 39). When the gp120 glycoproteins derived from different HIV-1 isolates are compared, five conserved regions (C1 to C5) and five variable regions (V1 to V5) can be identified (14, 42). The CD4 binding region of the HIV-1 gp120 glycoprotein is composed of discontinuous elements derived from the third and fourth conserved regions (26, 27, 35). Discontinuous epitopes overlapping the CD4 binding site serve as targets for a subset of neutralizing antibodies (4, 15, 20, 22, 23, 33, 38, 43, 46, 48, 49).

In addition to receptor binding, virus entry requires steps involved in the fusion of the viral and target cell membranes (17, 44). The details of the membrane fusion process are still obscure, but changes in the amino terminus, in the middle of the ectodomain, or in the membrane-spanning region of the gp41 transmembrane envelope glycoprotein can attenuate fusion capacity (3, 8, 9, 18, 25, 26, 45). It has also been reported previously that the HIV-1 gp41 glycoprotein can mediate membrane fusion in the absence of the gp120 glycoprotein (37). Genetic evidence suggests that the hydrophobic gp41 amino terminus interacts with the target cell during the fusion process (3).

Studies using site-directed mutagenesis and neutralizing antibodies have suggested that the gp120 glycoprotein also plays a role in the membrane fusion process. Amino acid changes in the V3 loop and in the C4 region can result in fusion-defective envelope glycoproteins (6, 10, 13, 17, 21, 36, 45, 50). Some antibodies against these regions neutralize HIV-1 and, for anti-V3 loop antibodies, neutralization occurs by the disruption of steps in virus entry other than receptor binding (30, 40).

The second variable (V2) region of the HIV-1 gp120 glycoprotein and the corresponding region of the simian immunodeficiency virus gp120 glycoprotein are also targets for neutralizing antibodies (11, 19, 23a). The V1 and V2 regions are contained in loops extending from a common stem stabilized by disulfide bonds (28). Monoclonal antibodies that recognize both conformation-dependent (G3-4) and conformation-independent (G3-136 and BAT-085) epitopes with V2 components can neutralize HIV-1 (11, 19). The BAT-085 and G3-136 antibodies recognize linear sequences (amino acids 169 to 183) within the V2 loop and neutralize HIV-1 without blocking receptor binding (11). The G3-136 antibody competes for gp120 binding with the G3-4 neutralizing antibody. The G3-4 antibody apparently recognizes a conformation-dependent epitope, since this antibody does not bind to gp120 glycoproteins that are denatured (19). Here we study the role of the V1 and V2 loops in HIV-1 envelope glycoprotein function and in recognition by the G3-4 neutralizing antibody.

Within the V1/V2 loops of HIV-1, relatively invariant amino acids are interspersed among highly variable residues. Single or pairwise amino acid changes were introduced into the more conserved V1/V2 amino acids of the HXBc2 envelope glycoprotein, which is derived from the human T-cell lymphotropic virus IIIB (HTLV-IIIB) strain of HIV-1 (12). The pSVIIIenv plasmid expressing the wild-type or mutant envelope glycoproteins was cotransfected into COS-1 cells along with a plasmid expressing the HIV-1 *tat* protein, which increases the level of envelope glycoprotein expression in this system. The radiolabeled envelope proteins were examined by precipitation with a mixture of sera

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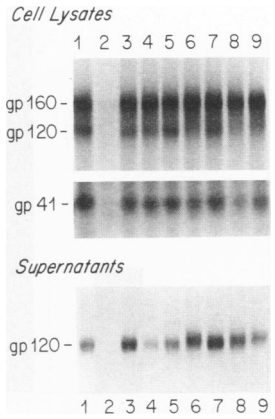

FIG. 1. Expression of mutant envelope glycoproteins in COS-1 cells. COS-1 cells were transfected with mutant glycoprotein expressor plasmids, labeled overnight with [^{35}S]cysteine, and lysed in RIPA buffer. Labeled HIV-1 envelope glycoproteins were immunoprecipitated with polyclonal, HIV-1-positive patient sera. Lanes: 1, wild-type HXBc2 glycoproteins; 2, ΔKS (envelope deletion); 3, 152/153 GE/SM; 4, 166 R/L; 5, 168 K/L; 6, 176/177 FY/AT; 7, 179/180 LD/DL; 8, 183/184 PI/SG; 9, 191/192/193 YSL/GSS.

from AIDS patients as described previously (48). For three of the mutants (176/177 FY/AT, 183/184 PI/SG, and 191/192/193 YSL/GSS), decreases in the amounts of cell-associated gp120 and gp41 glycoproteins relative to those of the wild-type glycoproteins were observed, even though the levels of cell-associated gp160 precursor proteins were comparable to those of the wild-type glycoproteins (Fig. 1). The level of gp120 glycoprotein present in the supernatants for each of these mutants was comparable to or greater than that of the wild-type glycoproteins. These results suggest that these mutant gp120 molecules did not efficiently associate with the gp41 glycoprotein following cleavage of the gp160 precursor protein. A change from arginine to leucine at position 166 (166 R/L) in the V2 loop yielded a glycoprotein that exhibited a mild decrease in the rate of processing of the gp160 precursor. This conclusion follows from the decreased levels of both cell-associated and supernatant gp120 glycoprotein observed for this mutant relative to those of the wild-type glycoproteins.

For three mutants, a significant amount of native gp120 glycoprotein was observed in the cell lysates. One amino acid change in the V2 loop, 168 K/L, resulted in a mutant envelope glycoprotein exhibiting wild-type levels of precursor processing and subunit association. Two mutants (152/153 GE/SM, affecting the carboxyl terminus of the V1 region, and 179/180 LD/DL in the V2 loop) exhibited slight decreases in the ratio of cell-associated gp120 to supernatant gp120 relative to that seen for the wild-type glycoproteins, indicating a mild decrease in the affinity of the gp120-gp41 interaction for these mutants. The level of envelope glycoprotein expression on the COS-1 cell surface for the three mutants was examined by iodination of the transfected COS-1 cells by the lactoperoxidase method, followed by precipitation with a mixture of sera from AIDS patients as described previously (51). The results shown in Fig. 2 demonstrate that the amount of the gp120 envelope glycoprotein on the surface of COS-1 cells expressing the 152/153 GE/SM, 168 K/L, and 179/180 LD/DL proteins was at least as great as that of the wild-type glycoproteins. As has been previously observed for *tat*-enhanced envelope glycoprotein

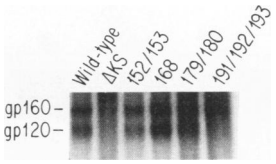

FIG. 2. Cell surface expression of mutant envelope glycoproteins. COS-1 cells were transfected with mutant envelope expressor plasmids. Total cell surface proteins were labeled with ^{125}I with lactoperoxidase (51), and cells were lysed in RIPA buffer. Envelope glycoproteins were precipitated with a mixture of HIV-1-positive patient sera. 152/153, 152/153 GE/SM; 168, 168 K/L; 179/180, 179/180 LD/DL; 191/192/193, 191/192/193 YSL/GSS.

expression in COS-1 cells, the gp160 glycoprotein precursor was also detected on the COS-1 cell surface. The gp160 envelope glycoprotein was the predominant form detected on the surface of COS-1 cells expressing the 191/192/193 YSL/GSS mutant that was defective in subunit association.

The abilities of COS-1 cells expressing the wild-type and mutant glycoproteins to nucleate the formation of syncytia upon cocultivation with SupT1 CD4-positive lymphocytes was examined as previously described (29, 41, 47). The data in Table 1 indicate that all of the V1/V2 mutants were significantly less efficient than the wild-type envelope glycoproteins in syncytium formation. The 166 R/L, 176/177 FY/AT, 183/184 PI/SG, and 191/192/193 YSL/GSS mutants did not induce cell fusion, as expected from the defects in processing or subunit association observed for these mutants. The syncytium-forming abilities of the 152/153 GE/SM, 168 K/L, and 179/180 LD/DL mutants were all less than 30% that of the wild-type envelope glycoproteins.

To examine whether the reduced syncytium-forming abilities of the 152/153 GE/SM, 168 K/L, and 179/180 LD/DL mutants might result from changes in their abilities to bind the CD4 receptor, two assays were utilized. In the first assay, radiolabeled soluble gp120 shed spontaneously into the transfected COS-1 cell supernatants was tested for its ability to bind to CD4-positive SupT1 lymphocytes (35). In this assay, all three mutant glycoproteins bound to SupT1

TABLE 1. CD4-binding and syncytium-forming abilities of V1/V2 loop mutant glycoproteins[a]

Envelope mutant	CD4-binding ability[b]	Syncytium-forming ability[c]
Wild type	1.0	100
152/153 GE/SM	0.64	9
166 R/L	0.44	4
168 K/L	0.94	28
176/177 FY/AT	0.17	10
179/180 LD/DL	0.96	24
183/184 PI/SG	0.10	12
191/192/193 YSL/GSS	0.25	6

[a] COS-1 cells were transfected with either wild-type or mutant envelope glycoprotein expressor plasmids.

[b] To measure CD4-binding abilities, transfected COS cells were labeled overnight with [^{35}S]cysteine. Labeled gp120 was harvested from the COS cell supernatant and bound to CD4-positive SupT1 cells. Bound and free gp120 glycoproteins were precipitated with a mixture of HIV-1-positive patient sera, and the binding ability was calculated as follows: CD4 binding ability = (bound mutant gp120/free mutant gp120) × (free wild-type gp120/bound wild-type gp120).

[c] To measure syncytium-forming abilities, COS cells expressing envelope glycoproteins were placed in suspension and cocultivated with SupT1 cells as described previously (3). Syncytia were scored after incubation for 8 to 12 h.

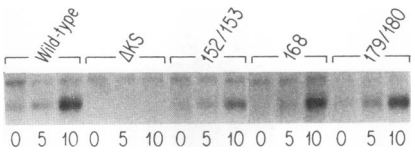

FIG. 3. Soluble CD4-induced shedding of mutant glycoproteins. Glycoproteins expressed in transfected COS-1 cells were labeled with [^{35}S]cysteine. Cells were incubated at 37°C for 90 min with 0, 5, and 10 μg of soluble CD4 in 1 ml of phosphate-buffered saline. Glycoproteins from the supernatant were precipitated with a mixture of HIV-1-positive patient sera. For explanations of glycoprotein designations, see the legend to Fig. 2.

cells, with only a slight reduction in the binding of the 152/153 GE/SM mutant (Table 1). In a second assay, the sensitivity of the multimeric envelope glycoprotein complex to soluble CD4-induced shedding of the gp120 exterior glycoprotein (5, 16, 34, 45) was assessed (Fig. 3). The 152/153 GE/SM, 168 K/L, and 179/180 LD/DL mutants all exhibited gp120 shedding in response to soluble CD4. At concentrations of soluble CD4 up to 10 μg/ml, the amounts of shedding of the 168 K/L and 179/180 LD/DL mutants were similar to that of the wild-type envelope glycoprotein. Very slight decreases in the efficiency of shedding of the 152/153 GE/SM gp120 glycoprotein were consistent with the mild decrease in binding of the monomeric soluble gp120 glycoprotein of this mutant to CD4-positive cells.

To assess whether the glycoprotein mutants exhibiting substantial precursor processing and subunit association could mediate virus entry, the abilities of the mutants to complement the replication of an *env*-defective provirus expressing the bacterial chloramphenicol acetyltransferase gene was assessed (17). The 152/153 GE/SM and 179/180 LD/DL mutants exhibited reductions in their abilities to mediate entry into Jurkat or SupT1 lymphocytes (Fig. 4), relative to that of the wild-type glycoproteins. The ability of the 168 K/L mutant to complement virus replication was comparable to that of the wild-type envelope glycoproteins.

Previous studies suggested that some of the neutralizing antibodies, like G3-4, that compete with anti-V2 loop antibodies for gp120 binding recognize conformation-dependent structures (19). Since the G3-4 epitope could not be defined with peptides (19), we used a panel of HIV-1 gp120 mutants to determine whether particular amino acid changes in the molecule would affect the ability of the G3-4 antibody to precipitate the gp120 glycoprotein. Radiolabeled lysates in radioimmunoprecipitation assay (RIPA) buffer from transfected COS-1 cells were precipitated either with a mixture of sera from AIDS patients or with the G3-4 antibody as described previously (48). Since the mixture of sera reacts with multiple gp120 regions, an indication of the amount of mutant glycoprotein present in the cell lysates can be obtained. Specific defects in the recognition of particular mutant glycoproteins by the G3-4 antibody were observed. Table 2 shows that the vast majority of envelope glycoprotein mutants were precipitated comparably to the wild-type glycoprotein by the G3-4 antibody. Deletion of the V1/V2 loop resulted in the loss of G3-4 recognition, even though this deleted molecule is still recognized by several conformation-dependent antibodies and retains CD4-binding ability (35, 46, 48). Complete loss of G3-4 recognition was observed for the 176/177 FY/AT and 179/180 LD/DL mutants. The 183/184 PI/SG mutant was very poorly recognized by the

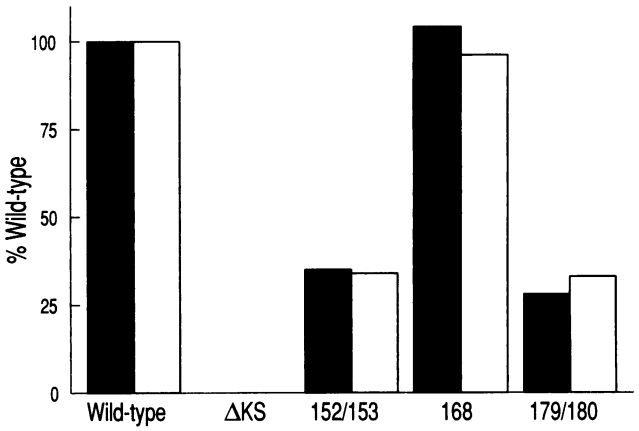

FIG. 4. Ability of mutant glycoproteins to mediate entry into Jurkat and SupT1 lymphocytes. Mutant envelope expressor plasmids were cotransfected into COS-1 cells with envelope-deleted chloramphenicol acetyltransferase provirus as described previously (17). Virions containing wild-type or mutant envelope glycoproteins were harvested from COS cell supernatants 36 h posttransfection, normalized for reverse transcriptase activity, and used to infect either Jurkat (solid bars) or SupT1 (open bars) CD4-positive lymphocytes. Expression of the chloramphenicol acetyltransferase gene represents a single round of viral replication in the target lymphocytes. The percent conversion of chloramphenicol is expressed as a percentage of the conversion with wild-type envelope (wild type equals 100%). For explanations of glycoprotein designations, see the legend to Fig. 2.

G3-4 antibody, and the recognition of the 191/192/193 YSL/GSS mutant was significantly reduced compared with that of the wild-type glycoproteins. The other mutants with residue changes within the V1 or V2 loop were precipitated by the G3-4 antibody comparably to the wild-type glycoproteins. Slight reductions in G3-4 recognition were observed for two of the mutants with amino acid changes near the base of the V1/V2 loop (117 K/W and 207 K/W). Other mutants with changes outside of the V1/V2 loop exhibited slight reductions in G3-4 recognition (e.g., 88N/P and 262 N/T), but these amino acid changes have been previously shown to conformationally disrupt a fraction of the envelope glycoproteins present in COS-1 cell lysates (35).

The studies reported herein provide insight into the functional importance of the gp120 V1/V2 regions. The variability of these regions is confined to specific amino acids that are interspersed with conserved residues. Changes in the conserved residues resulted in envelope glycoproteins with reduced functions. The reduced functions of the 176/177 FY/AT, 183/184 PI/SG, and 191/192/193 YSL/GSS mutants apparently result from disruption of the noncovalent association between gp120 and gp41 subunits. Subunit dissociation has been observed for mutants containing amino acid insertions in the V1 or V2 stem-loop structure (26). Other studies have demonstrated that the V1 and V2 loop can be deleted without disrupting gp120-gp41 association (50a). Taken together, the data suggest that inappropriate conformations of the V1 or V2 loop can interfere with subunit association, although the V1/V2 sequences are not required for gp120-gp41 binding. The proximity of parts of the V1/V2 loop to the gp120 or gp41 regions important for subunit association could explain these observations.

Three of the mutants (152/153 GE/SM, 168 K/L, and 179/180 LD/DL) exhibited decreases in syncytium-forming

TABLE 2. Precipitation of HIV-1 gp120 mutants from COS-1 cell lysates by the G3-4 antibody[a]

Envelope glycoprotein	RI[b]
Wild type	++++
88 N/P	+++
117 K/W	+++
Δ119-205	−
152/153 GE/SM	+++
166 R/L	++++
168 K/L	++++
176/177 FY/AT	−
179/180 LD/DL	−
183/184 PI/SG	+
191/192/193 YSL/GSS	++
207 K/W	+++
256 S/Y	+++
262 N/T	++
381 E/P	+++
438 P/R	+++

[a] Transfected COS-1 cells were lysed in RIPA buffer and precipitated with the G3-4 antibody or with a mixture of HIV-1-positive patient sera.

[b] The recognition index (RI) represents the following ratio:

$$RI = \left(\frac{\text{mutant gp160 + gp120}}{\text{wild-type gp160 + gp160}}\right) \times \left(\frac{\text{wild-type gp160 + gp120}}{\text{mutant gp160 + gp120}}\right)$$

The first set of data in parentheses applies to G3-4 antibody, while the second set applies to patient sera. RIs are expressed as follows: ++++, RI ≥ 0.90; +++, 0.60 ≤ RI < 0.90; ++, 0.16 ≤ RI < 0.60; +, 0.05 ≤ RI < 0.16; and −, no detectable recognition by antibody. The following mutants exhibited RIs greater than 0.90: 40 Y/D, 69 W/L, 80 N/R, 102 E/L, 103 Q/F, 106 E/A, 113 D/A, 113 D/R, 120/121 VK/LE, 125 L/G, 252 R/W, 257 T/R, 257 T/A, 257 T/G, 266 A/E, 267 E/L, 269 E/L, 281 A/V, 298 R/G, 308/309/310 RIQ/RPELIPVQ, 314 G/W, Δ297-329, 356 N/I, 368 D/R, 368 D/P, 368 D/T, 370 E/Q, 370 E/R, 370 E/K, 370 E/D, 380/381 GE/YV, 382 F/L, 384 Y/E, 386 N/Q, 392 N/E 397 N/E, 395 W/S, 406 N/G, 420 I/R, 421 K/L, 427 W/V, 427 W/S, 429 K/L, 430 V/S, 432 K/A, 433 A/L, 435 Y/H, 435 Y/S, 450 T/N, 456 R/K, 457 D/A, 457 D/G, 457 D/R, 457 D/N, 457 D/E, 463 N/D, 465 S/L, 470 P/G, 475 M/S, 477 D/V, 485 K/V, 491 I/F, 493 P/K, 495 G/K, 497/498/499 APT/VLL, and 500/501 KA/KGIPKA.

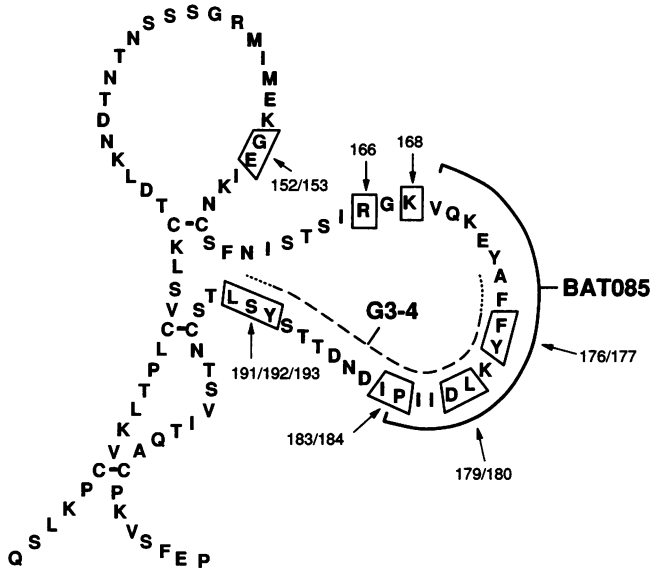

FIG. 5. V1/V2 loops of gp120. Boxed amino acids represent residues mutated for functional analysis of the V1/V2 loops. Epitopes for neutralizing antibodies BAT-085 (solid line) and G3-4 (broken line) were defined by peptide mapping (19) and genetic analysis. 166, 166 R/L; 176/177, 176/177 FY/AT; 183/184, 183/184 PI/SG. For other explanations of glycoprotein designations, see the legend to Fig. 2.

ability not explained by changes in the level of cell surface envelope glycoprotein expression. While the 152/153 GE/SM mutant exhibited a slight decrease in CD4-binding ability, this decrease is not sufficient to account for the observed reduction in cell-fusing capacity (47). The efficient shedding of the exterior glycoprotein at subsaturating concentrations of soluble CD4 suggests that the affinity of the multimeric envelope glycoprotein complex for CD4 is not altered by the 168 K/L or 179/180 LD/DL changes (45). Therefore, these two mutants, and probably the 152/153 GE/SM mutant as well, exhibit defects in the membrane fusion process. The results provide additional examples emphasizing the lack of correlation between fusion capacity and soluble CD4-induced gp120 shedding (45). The decreased abilities of the 152/153 GE/SM and 179/180 LD/DL mutants to mediate virus entry are also consistent with a fusion defect. This interpretation is supported by our observation that the level of virion-associated gp120 glycoprotein is comparable for the wild-type, 152/153 GE/SM, and 179/180 LD/DL glycoproteins (data not shown). As has been observed for other HIV-1 envelope glycoprotein mutants with attenuated membrane-fusing capacity (25), syncytium-forming ability appears to be more sensitive to the 168 K/L change than does the ability to complement virus replication. This difference may result from the different number of successful envelope glycoprotein-receptor interactions required for cell-cell fusion relative to virus entry (25).

Some of the amino acid changes in the V2 loop resulted in moderate decreases in CD4-binding ability (Table 1). This subset of V2 mutants also exhibited decreases in their abilities to associate with the gp41 glycoprotein, suggesting that some conformational disruption results from these amino acid alterations. The effect of major conformational changes in V2 loop structure on CD4 binding may relate to the observation that some antibodies, like G3-4, that recognize determinants within the V2 loop weakly compete with CD4 for gp120 binding (11). Future studies will be required in order to define the relationship of the V2 loop and CD4 binding region of the HIV-1 gp120 glycoprotein.

The amino acid changes that significantly affect the binding of the conformation-dependent neutralizing antibody, G3-4, are all located within the carboxyl half of the V2 loop. This result is consistent with the ability of the G3-4 antibody to compete for gp120 binding with other antibodies that recognize linear V2 sequences. These mapping studies suggest that amino acids critical for G3-4 recognition reside within V2 residues 176 to 184. Amino acid changes outside the V2 loop did not affect G3-4 recognition dramatically. The G3-4 antibody recognition site may be composed of elements derived from the V2 loop that assume a conformation not readily mimicked by peptides or denatured gp120 protein. This interpretation is supported by the observation that a chimeric Moloney murine leukemia virus envelope protein containing the V1/V2 loop of HIV-1 can be recognized by the G3-4 antibody (37a).

The G3-4 epitope was disrupted by endoglycosidase H treatment of the gp120 glycoprotein (19). There is an N-linked glycosylation site at asparagine 186 within the G3-4 epitope mapped in this study. However, in Chinese hamster ovary cells, the added sugars at this site represent complex, and therefore endoglycosidase H-resistant, forms (28). Disruption of gp120 recognition by the G3-4 antibody following endoglycosidase H treatment may result from the effects of carbohydrate modification distant from the epitope. Further

work will be required in order to determine the role of particular carbohydrate moieties in the formation of the G3-4 epitope.

A summary of the results, shown in Fig. 5, indicates that neutralizing antibodies can recognize either the amino or the carboxyl side of the V2 loop, with overlap of the epitopes near the tip of the loop. Amino acid changes in the tip of the V2 loop, such as 179/180 LD/DL, result in fusion-deficient phenotypes. This situation is reminiscent of that found with the gp120 V3 loop. In general, however, the sensitivity of HIV-1 envelope glycoprotein function to amino acid changes or antibody binding in the V2 loop appears to be less than that previously observed for the V3 loop. Further work should determine the feasibility of using the V2 loop as a target for intervention in the HIV-1 infection process.

We thank Virginia Nixon and Leroy D. Johnson for manuscript preparation and Amy Emmert for artwork.

Markus Thali was supported by the Swiss National Science Foundation. David D. Ho was supported by NIH grants AI25541, AI24030, and AI27742 and by the Aaron Diamond Foundation. Joseph Sodroski was supported by the Leukemia Society of America and by the NIH (grants AI24755 and AI31783). Both the Aaron Diamond AIDS Research Center and the Dana-Farber Cancer Institute are recipients of Centers for AIDS Research Awards from the National Institutes of Health.

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