JOURNAL OF VIROLOGY, Dec. 1990, p. 5701–5707
0022-538X/90/125701-07$02.00/0
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Vol. 64, No. 12

Identification of Individual Human Immunodeficiency Virus Type 1 gp120 Amino Acids Important for CD4 Receptor Binding

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Received 23 May 1990/Accepted 24 August 1990

The binding of the CD4 receptor by the human immunodeficiency virus type 1 gp120 exterior envelope glycoprotein is important for virus entry and cytopathic effect. To investigate the CD4-binding region of the gp120 glycoprotein, we altered gp120 amino acids, excluding cysteines, that are conserved among the primate immunodeficiency viruses utilizing the CD4 receptor. Changes in two hydrophobic regions (Thr-257 in conserved region 2 and Trp-427 in conserved region 4) and two hydrophilic regions (Asp-368 and Glu-370 in conserved region 3 and Asp-457 in conserved region 4) resulted in significant reductions in CD4 binding. For most of the mutations affecting these residues, the observed effects on CD4 binding did not apparently result from global conformational disruption of the gp120 molecule, as assessed by measurements of precursor processing, subunit association, and monoclonal antibody recognition. The two hydrophilic regions exhibit a strong propensity for β-turn formation, are predicted to act as efficient B-cell epitopes, and are located adjacent to hypervariable, glycosylated regions. This study defines a small number of gp120 residues important for CD4 binding, some of which might constitute attractive targets for immunologic intervention.

Human immunodeficiency virus type 1 (HIV-1) is the etiologic agent of acquired immune deficiency syndrome (4, 12, 30), which is characterized by depletion of CD4-positive lymphocytes (13, 21). The tropism of HIV-1 for CD4-positive cells is due to a specific interaction between CD4, the viral receptor, and the gp120 exterior envelope glycoprotein (10, 16, 17, 24). After receptor binding, the viral envelope glycoproteins gp120 and gp41 mediate the fusion of the viral and host cell membranes to allow viral entry (33). The gp120 and gp41 glycoproteins are derived from the cleavage of a gp160 envelope glycoprotein precursor and are held together by noncovalent interactions (1, 18, 31).

Insertions or deletions in conserved gp120 regions C1, C3, C4, and C5 have been shown to affect CD4 binding (7, 8, 18, 22, 23, 35), although the effect of these changes on gp120 conformation was not examined. A proteolytic fragment composed of the 160 carboxy-terminal gp120 residues has been reported to bind CD4 (28), and antibodies directed against C4 or C5 could block CD4 binding in some circumstances (2, 11, 22, 34). To define the CD4-binding region more precisely, we altered gp120 amino acids, excluding cysteines, that are conserved among the primate immunodeficiency viruses utilizing the CD4 receptor (27). We examined the processing of the gp160 precursor, the association of the gp120 and gp41 glycoproteins, and recognition of the mutant gp120 molecules by monoclonal antibodies to assess the potential effects of the introduced changes on gp120 conformation. The results define four gp120 regions that are critical for the interaction with CD4. Two of the regions are predicted to be exposed on the native molecule and therefore might constitute attractive targets for immunologic intervention.

MATERIALS AND METHODS

Construction of plasmids expressing mutant envelope glycoproteins. The *Kpn*I-*Bam*HI fragment of the pSVIIIenv plasmid (14) was used for site-directed mutagenesis by the procedure of Kunkel et al. (19). The presence of the mutation was confirmed by the generation of a novel restriction endonuclease site in some cases and by DNA sequencing (32). Two independent clones of each mutated *env* fragment were prepared and used for the CD4 binding assay to ensure that spontaneous mutations distant from the desired mutation were not responsible for the observed phenotypes.

Measurement of CD4-binding ability of mutant glycoproteins. COS-1 cells were transfected by the DEAE-dextran procedure (9) with 10 μg of pSVIIIenv plasmid containing either the wild-type or mutated HXBc2 *env* gene. At 48 h after transfection, cells were labeled with [^{35}S]cysteine (50 μCi/ml). Labeled supernatants were incubated with 5×10^7 SupT1 lymphocytes at 37°C for 1 h. The SupT1 cells were washed once with phosphate-buffered saline, lysed in 1.0 ml of RIPA lysis buffer, and used for immunoprecipitation with excess 19501 AIDS patient serum as described previously (14). Precipitates were analyzed on sodium dodecyl sulfate-polyacrylamide gels, and the autoradiograms were quantitated by densitometry. Pilot studies demonstrated that gp120 binding to SupT1 lymphocytes was completely blocked by OKT4a monoclonal antibody, indicating dependence on the CD4 molecule.

The relative CD4-binding ability of gp120 mutants was calculated by the following formula:

$$\text{Relative binding ability} = \frac{[\text{gp120 bound}]_{\text{mutant}} \times [\text{gp120 free}]_{\text{wild type}}}{[\text{gp120 free}]_{\text{mutant}} \times [\text{gp120 bound}]_{\text{wild type}}}$$

RESULTS

Effect of changes in gp120 on CD4 binding. Amino acids that were altered in this study are shown in Table 1. These residues were selected because they either did not vary or exhibited strong conservation of features among HIV-1,

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TABLE 1. CD4-binding ability of HIV-1 gp120 mutants

Amino acid change[a]	Relative CD4-binding ability[b]
Wild type	1.00
36 V/L	1.44 ± 0.12
40 Y/D	1.23 ± 0.10
45 W/S	0.84 ± 0.08
69 W/L	1.36 ± 0.15
76 P/Y	1.36 ± 0.20
76 P/N	1.16 ± 0.21
80 N/R	0.62 ± 0.08
83 E/R	NP[c]
83 E/Y	NP
88 N/P	0.89 ± 0.09
91 E/R	1.21 ± 0.07
93/94 FD/TR	NP
102 E/L	0.82 ± 0.09
103 Q/F	0.78 ± 0.11
106 E/A	1.53 ± 0.15
113 D/A	1.16 ± 0.09
113 D/R	0.85 ± 0.06
117 K/W	1.06 ± 0.08
120/121 VK/LE	0.51 ± 0.04
125 L/G	1.31 ± 0.23
207 K/W	1.02 ± 0.10
227 K/E	NP
252 R/W	2.5 ± 0.29
256 S/Y	0.30 ± 0.07
256 S/R	NP
257 T/R	0.16 ± 0.03
257 T/A	1.12 ± 0.20
257 T/G	1.04 ± 0.15
259 L/K	NP
262 N/T	0.21 ± 0.04
266 A/E	0.97 ± 0.08
267 E/L	0.76 ± 0.11
269 E/L	0.61 ± 0.10
298 R/G	1.00 ± 0.07
314 G/W	0.54 ± 0.10
368 D/R	<0.004
368 D/P	0.09 ± 0.02
368 D/T	0.33 ± 0.05
368 D/N	0.019 ± 0.003
368 D/K	<0.005
368 D/E	0.09 ± 0.01
370 E/Q	0.018 ± 0.002
370 E/R	<0.003
370 E/D	0.45 ± 0.07
377 N/K	0.69 ± 0.09
380 G/F	0.78 ± 0.07
381 E/P	1.09 ± 0.09
382 F/L	2.7 ± 0.5
384 Y/E	0.29 ± 0.03
391 F/Q	NP
395 W/S	1.11 ± 0.13
420 I/R	1.24 ± 0.11
421 K/L	0.55 ± 0.05
427 W/V	<0.006
427 W/S	<0.012
429 K/L	1.07 ± 0.16
430 V/S	0.39 ± 0.05
432 K/A	1.62 ± 0.25
433 A/L	1.66 ± 0.25
435 Y/H	1.43 ± 0.13
435 Y/S	0.77 ± 0.07
438 P/R	2.3 ± 0.3
447 S/I	0.27 ± 0.04
457 D/A	0.09 ± 0.01
457 D/R	0.15 ± 0.04
463 N/D	1.2 ± 0.01
470 P/L	0.54 ± 0.007
474 D/A	1.01 ± 0.22
475 M/S	1.03 ± 0.03
476 R/D	0.71 ± 0.007
477 D/R	NP
477 D/V	0.39 ± 0.06
477 D/S	0.53 ± 0.07
482/483/484 ELY/GRA	0.44 ± 0.05
485 K/V	0.79 ± 0.08
486/487 YK/WP	NP
491 I/F	1.28 ± 0.13
493 P/K	1.78 ± 0.20
495 G/K	1.71 ± 0.18
497/498/499 APT/VLL	0.98 ± 0.09
500/501 KA/KGIPKA	0.91 ± 0.11

[a] The number of the mutant refers to the envelope glycoprotein amino acid residue of the HXBc2 strain of HIV-1, where 1 is the initial methionine (27). The mutations result in substitution of the amino acid(s) on the right for the amino acid(s) on the left; for example, 273 R/I indicates a substitution of isoleucine for the arginine residue at position 273.

[b] The relative CD4-binding ability was calculated as described in Materials and Methods. The standard deviation is also indicated.

[c] Inefficient processing of the gp160 precursor to gp120 and gp41 glycoproteins was observed for these mutants. CD4-binding ability was not determined.

HIV-2, simian immunodeficiency virus SIV_{mac}, and SIV_{agm} gp120 exterior envelope glycoproteins (27, 36). The mutated *env* gene of the HXBc2 HIV-1 strain was cloned into the pSVIIIenv plasmid, which allows a high level of transient expression of the gp160 envelope precursor in transfected COS-1 cells (14). For gp160 mutants that undergo proteolytic cleavage, the mature gp120 exterior envelope glycoprotein can be detected in supernatants of transfected COS-1 cells, due to the lability of gp120 association with the gp41 transmembrane glycoprotein (14, 18). Radiolabeled gp120 present in transfected COS-1 cell supernatants was used to assess the ability to bind to the CD4 molecule on the surface of SupT1 lymphocytes. All the CD4 binding experiments were performed under conditions in which the CD4 concentration was not limiting for gp120 binding (data not shown) so that the calculated relative binding ability approximated the true ratio of the mutant/wild-type binding constants. Pilot experiments indicated that, over a greater than 20-fold range of total gp120 concentrations, the ratio of bound/free gp120 did not vary for the wild-type glycoprotein (data not shown).

Figure 1A shows several examples of the results, which were used to calculate the CD4-binding abilities of the mutant gp120 glycoproteins (Table 1 and Fig. 1B). With nine exceptions, all the mutant envelope glycoproteins were processed to gp120 molecules detected in the COS-1 cell supernatants. Even though most of the introduced amino acid changes were not conservative, the majority of gp120 mutants exhibited CD4-binding ability that did not differ more than twofold from that of the wild-type glycoprotein. These results indicate that the majority of well-conserved gp120 residues are not, per se, essential for high-affinity CD4 binding.

Substitution mutations affecting 12 gp120 residues resulted in glycoproteins that exhibited less than 50% of the CD4-binding ability of the wild-type gp120. The effects of the introduced changes on the processing of the gp160 precursor, the association of the gp120 molecule with the gp41 glycoprotein on the expressing cell, and the ability of three mouse monoclonal antibodies (HA1, HA2, and HT2) that recognize conformation-dependent gp120 epitopes (David

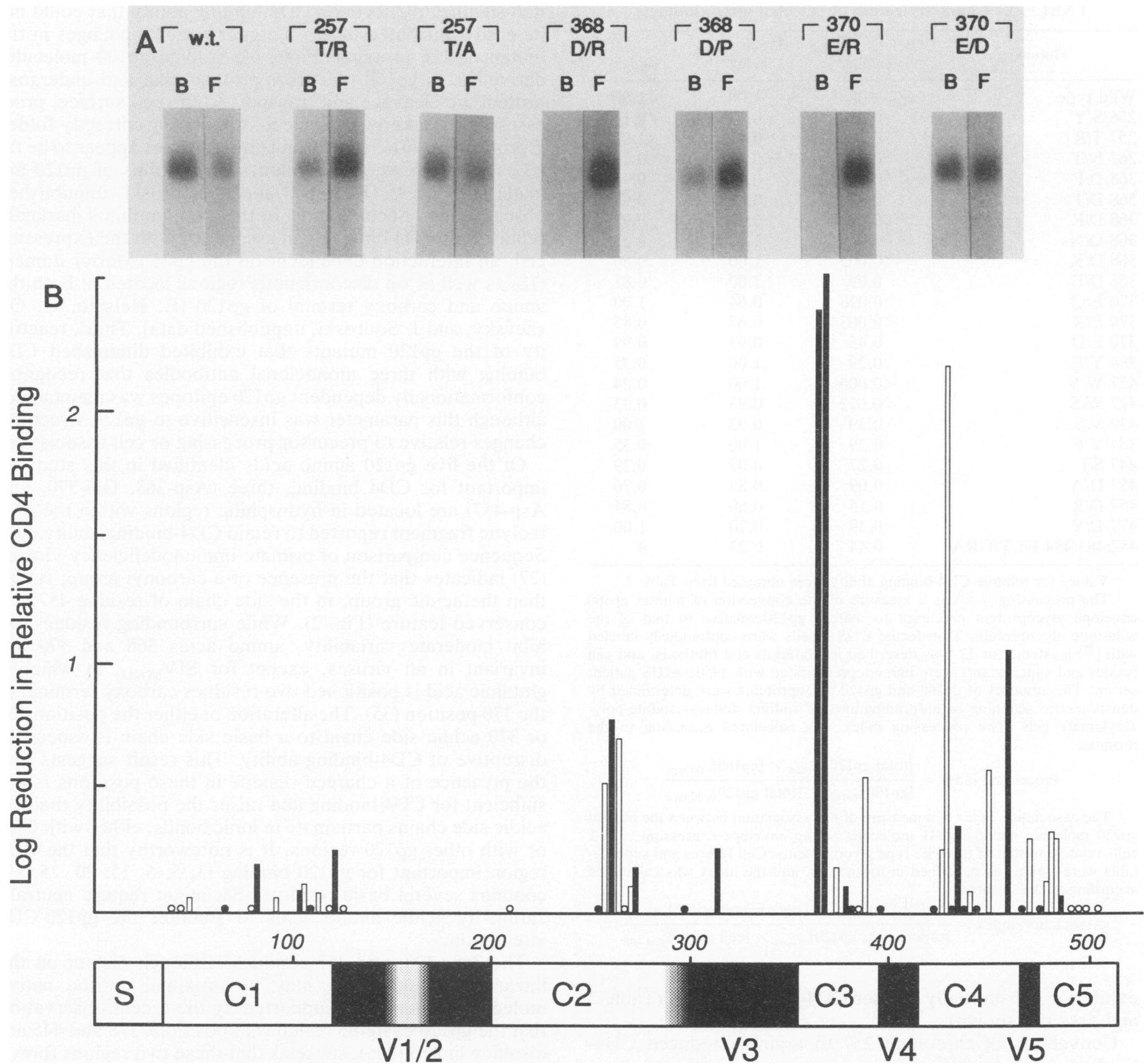

FIG. 1. Relative CD4-binding ability of gp120 mutants. (A) Amount of gp120 bound (lanes B) to the surface of SupT1 lymphocytes, as well as the unbound gp120 (lanes F), is shown for wild-type (w.t.) and mutant glycoproteins. (B) Log_{10} of the reduction in relative CD4-binding ability observed for the most disruptive change at a given amino acid residue. The open bars indicate mutant glycoproteins that exhibited processing or association indices less than 40% those of the wild-type glycoproteins. The solid bars represent mutant glycoproteins for which both processing and association indices were at least 40% of the wild-type values. The linear sequence of the HIV-1 gp120 molecule is shown, with the conserved regions unshaded and the variable regions shaded dark. Numbers indicate amino acid residues. S, Signal sequence.

Ho, unpublished data) to precipitate the mutant glycoprotein were examined (Table 2). Several of the mutants (256 S/Y, 262 N/T, 384 Y/E, 427 W/V, 427 W/S, 447 S/I, 477 D/V, and 482/483/484 ELY/GRA) exhibited greater than 60% reduction in either precursor processing or cell association, which suggested that significant local or global conformational changes in gp120 resulted from these amino acid alterations. Recognition of the wild-type and these mutant gp120 molecules by the monoclonal antibodies was similar (data not shown), suggesting that gp160 processing and cell association of gp120 were more sensitive indicators of changes in envelope structure.

The most significant decreases in CD4-binding ability were observed for changes involving Thr-257, Asp-368, Glu-370, Trp-427, and Asp-457. A greater than 100-fold reduction in CD4-binding ability resulted from some of the mutations affecting Asp-368, Glu-370, or Trp-427. An approximately 10-fold reduction in CD4-binding ability was observed for some changes in Thr-257 or Asp-457. While the changes in Trp-427 significantly affected association with the gp41 envelope glycoprotein, the effects of changes in the other four residues on gp160 processing and cell association of gp120 were small compared with those observed for other mutants

TABLE 2. Characterization of selected gp120 mutants

Mutant	Relative CD4 binding[a]	Processing index[b]	Association index[c]
Wild type	1.00	1.00	1.00
256 S/Y	0.30	0.17	0.17
257 T/R	0.16	0.43	1.0
262 N/T	0.21	0.07	0.14
368 D/P	0.09	0.94	0.91
368 D/T	0.33	0.86	0.93
368 D/R	<0.004	0.79	0.97
368 D/N	0.019	1.00	0.51
368 D/K	<0.005	1.00	0.60
368 D/E	0.09	1.00	0.81
370 E/Q	0.018	0.64	1.00
370 E/R	<0.003	0.67	0.85
370 E/D	0.45	0.93	0.99
384 Y/E	0.29	1.00	0.35
427 W/V	<0.006	1.00	0.24
427 W/S	<0.012	0.93	0.33
430 V/S	0.39	0.93	1.00
384 Y/E	0.29	1.00	0.35
447 S/I	0.27	0.07	0.29
457 D/A	0.09	0.88	0.76
457 D/R	0.15	0.60	0.84
477 D/V	0.39	0.20	1.00
482/483/484 ELY/GRA	0.44	0.23	0

[a] Values for relative CD4-binding ability were obtained from Table 1.

[b] The processing index is a measure of the conversion of mutant gp160 envelope glycoprotein precursor to mature gp120 relative to that of the wild-type glycoprotein. Transfected COS-1 cells were continuously labeled with [^{35}S]cysteine for 12 h as described in Materials and Methods, and cell lysates and supernatants were immunoprecipitated with 19501 AIDS patient serum. The amounts of gp160 and gp120 glycoproteins were determined by densitometric scanning of autoradiograms of sodium dodecyl sulfate-polyacrylamide gels. The processing index was calculated according to the formula:

$$\text{Processing index} = \frac{[\text{total gp120}]_{\text{mutant}} \times [\text{gp160}]_{\text{wild type}}}{[\text{gp160}]_{\text{mutant}} \times [\text{total gp120}]_{\text{wild type}}}$$

[c] The association index is a measure of the association between the mutant gp120 molecule and the gp41 molecule on the envelope-expressing COS-1 cells relative to that of the wild-type glycoproteins. Cell lysates and supernatants were treated as described in footnote b, and the index was calculated according to the formula:

$$\text{Association index} = \frac{[\text{cell gp120}]_{\text{mutant}} \times [\text{supernatant gp120}]_{\text{wild type}}}{[\text{supernatant gp120}]_{\text{mutant}} \times [\text{cell gp120}]_{\text{wild type}}}$$

exhibiting substantially greater CD4-binding ability (Table 2 and data not shown).

Conversion of threonine 257 to arginine reduced CD4-binding ability by 84%, whereas more conservative changes to alanine or glycine resulted in a gp120 protein with nearly wild-type binding ability. These results indicate that some amino acid changes in this residue can dramatically disrupt CD4 binding, although the presence of threonine at position 257 is not indispensable for CD4 binding.

Some changes in the acidic residues in two regions of the gp120 glycoprotein, Asp-368/Glu-370 and Asp-457, resulted in marked reduction in CD4 binding. Complete loss of detectable CD4 binding was associated with conversion of residue 368 or 370 to a positively charged amino acid (368 D/R, 368 D/K, and 370 E/R). Decreases in binding affinity were noted for other changes in these residues, even for relatively conservative changes (368 D/E, 368 D/N, 370 E/D, and 370 E/Q).

DISCUSSION

Of the gp120 amino acids conserved among primate immunodeficiency viruses, alterations in five residues exhibited significant effects on CD4-binding ability that could not be easily attributed to gross conformational changes in the mutant gp120 proteins. First, we studied gp120 molecules derived from gp160 precursor proteins that had undergone proteolytic cleavage and transport to the cell surface, processes that are known to select strongly for correctly folded glycoproteins. Such constraints on transport appear to be far less restrictive when truncated soluble forms of gp120 are made (11, 18; M. Kowalski and J. Sodroski, unpublished observations). Second, most of the gp120 mutants markedly reduced for CD4 binding still associated with the expressing cell, an interaction dependent on the gp41 exterior domain (18) as well as on discontinuous regions located at both the amino and carboxy termini of gp120 (E. Helseth, U. Olshevsky, and J. Sodroski, unpublished data). Third, reactivity of the gp120 mutants that exhibited diminished CD4 binding with three monoclonal antibodies that recognize conformationally dependent gp120 epitopes was maintained, although this parameter was insensitive to gp120 structural changes relative to precursor processing or cell association.

Of the five gp120 amino acids identified in this study as important for CD4 binding, three (Asp-368, Glu-370, and Asp-457) are located in hydrophilic regions within the proteolytic fragment reported to retain CD4-binding ability (28). Sequence comparison of primate immunodeficiency viruses (27) indicates that the presence of a carbonyl group, rather than the acidic group, in the side chain of residue 457 is a conserved feature (Fig. 2). While surrounding residues exhibit moderate variability, amino acids 368 and 370 are invariant in all viruses, except for SIV_{MND}, in which a glutamic acid is positioned two residues carboxy terminal to the 370 position (35). The alteration of either the position 368 or 370 acidic side chain to a basic side chain is especially disruptive of CD4-binding ability. This result suggests that the presence of a charged residue in these positions is not sufficient for CD4 binding and raises the possibility that the acidic side chains participate in ionic bonds, either with CD4 or with other gp120 regions. It is noteworthy that the CD4 region important for gp120 binding (3, 5, 6, 15, 20, 25, 29) contains several basic residues that might require neutralization by acidic amino acids to stabilize the gp120-CD4 interaction.

The 368, 370, and 457 residues, although distant on the linear gp120 sequence, may be proximal on the native molecule. This model, supported by the recent observation that the gp120 cysteine residues at positions 378 and 445 are disulfide linked (22a), suggests that these two regions form a symmetrical structure (Fig. 3). The 368, 370, and 457 residues are located within hydrophilic regions exhibiting a strong potential to form β-turns (26), characteristics predictive of exposure on the surface of the native molecule. The hydrophilic properties and β-turn potential of these two regions suggest that they may be efficient B-cell epitopes and thus constitute important targets for immunoprophylaxis or immunotherapy. The immunogenicity of the position 368, 370, and 457 gp120 regions during natural HIV-1 infection may be modified by the proximity of these residues to highly variable regions (V3 and V5, respectively) that contain potential N-linked glycosylation sites (Fig. 3).

Antibodies directed against the hydrophobic gp120 region encompassing residues 423 to 438 interfere with CD4 binding (11, 22, 34). Changes in the most highly conserved amino acids within this region exert only small effects on CD4 binding, although we have confirmed that changes in the less well-conserved Trp-427 significantly affect CD4-binding ability (7). These changes also affect the ability of gp120 to

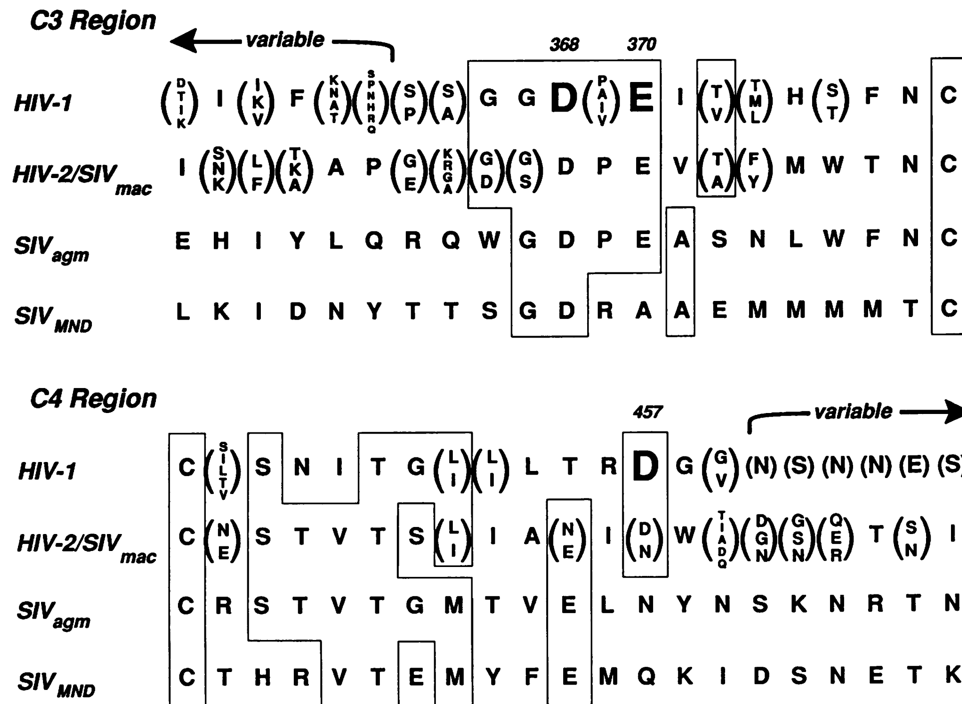

FIG. 2. Sequence comparison of primate immunodeficiency viruses (27, 35) near the 368, 370, and 457 residues (shown in boldface type). Identical amino acids are boxed, while different residues found at each position for different isolates are included in parentheses. Regions of hypervariability are indicated. For the C4 region of hypervariability in HIV-1, only the HXBc2 sequence is shown in parentheses because of the extreme degree of variability in this region. SIV, simian immunodeficiency virus.

efficiently associate with the gp41 transmembrane glycoprotein, suggesting that at least local conformational effects result from the mutations.

One mutation affecting gp120 residue 257, which is not contained within the gp120 proteolytic fragment reported to bind CD4 (28), significantly affects CD4 binding. Interpretation of this result awaits careful comparison of the relative CD4-binding affinity of the carboxy-terminal gp120 fragment and the complete gp120 moiety. It is possible that inappropriate changes in residues not per se involved in CD4 binding

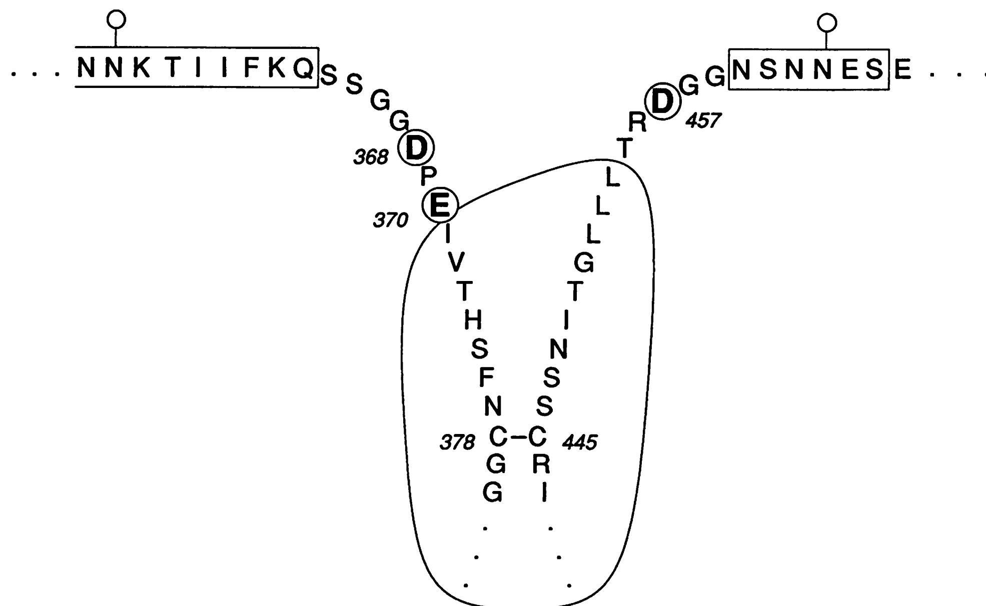

FIG. 3. Potential symmetrical structure formed by the C3 and C4 gp120 regions. The known disulfide bond between Cys-378 and Cys-445 (22a) occurs within a hydrophobic region encompassed by the solid line. Residues 368, 370, and 457 are contained within hydrophilic regions depicted outside the solid line. Sequences predicted to form β-turns (26) are illustrated as bends in the figure. Hypervariable regions (V3 and V5) are boxed, and sites of known glycosylation (22a) are depicted as ball-and-sticks.

but physically proximal to the CD4-binding region can diminish binding affinity. Alternatively, the region near Thr-257, as well as that near Trp-427, may contribute to hydrophobic interactions between gp120 and CD4. Further analysis will be required to distinguish which if any of the small group of gp120 amino acids identified as important for CD4 binding by mutagenesis directly interacts with CD4.

ACKNOWLEDGMENTS

We thank Robert Gallo, Flossie Wong-Staal, Max Essex, Bruce Walker, and David Ho for reagents; Jan Welch for manuscript preparation; and Amy Emmert for artwork. Udy Olshevsky performed this work while on sabbatical from the Israel Institute for Biological Research, Nes-Ziona, Israel.

Eirik Helseth was supported by a fellowship from the Norwegian Cancer Society. This work was supported by grants from the Leukemia Society of America and the Aaron Diamond Foundation and by Public Health Service grant AI24755 from the National Institutes of Health.

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