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Mutation at a Single Position in the V2 Domain of the HIV-1 Envelope Protein Confers Neutralization Sensitivity to a Highly Neutralization-Resistant Virus▽†

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Understanding the determinants of neutralization sensitivity and resistance is important for the development of an effective human immunodeficiency virus type 1 (HIV-1) vaccine. In these studies, we have made use of the swarm of closely related envelope protein variants (quasispecies) from an extremely neutralization-resistant clinical isolate in order to identify mutations that conferred neutralization sensitivity to antibodies in sera from HIV-1-infected individuals. Here, we describe a virus with a rare mutation at position 179 in the V2 domain of gp120, where replacement of aspartic acid (D) by asparagine (N) converts a virus that is highly resistant to neutralization by multiple polyclonal and monoclonal antibodies, as well as antiviral entry inhibitors, to one that is sensitive to neutralization. Although the V2 domain sequence is highly variable, D at position 179 is highly conserved in HIV-1 and simian immunodeficiency virus (SIV) and is located within the LDI/V recognition motif of the recently described α4β7 receptor binding site. Our results suggest that the D179N mutation induces a conformational change that exposes epitopes in both the gp120 and the gp41 portions of the envelope protein, such as the CD4 binding site and the MPER, that are normally concealed by conformational masking. Our results suggest that D179 plays a central role in maintaining the conformation and infectivity of HIV-1 as well as mediating binding to α4β7.

A major goal in human immunodeficiency virus type 1 (HIV-1) vaccine research is the identification of immunogens able to elicit protective immunity from HIV-1 infection. Results from the recent RV144 clinical trial in Thailand (53) have provided evidence that immunization with vaccines containing the recombinant HIV-1 envelope glycoprotein gp120 (6, 7) can protect humans from HIV infection when incorporated in a prime/boost immunization regimen. Although the level of protection observed in the RV144 trial (31%) was modest, it represents a significant advance in HIV-1 vaccine research and has rekindled the efforts to identify improved subunit vaccine antigens that might achieve even higher levels of protection. In these studies, we have sought to understand the molecular determinants of neutralization sensitivity and resistance in HIV-1 envelope proteins for the purpose of developing improved vaccine antigens.

In previous studies (47), we have described a novel method of mutational analysis of the HIV-1 envelope protein, termed swarm analysis, for identification of mutations that confer sensitivity and/or resistance to broadly neutralizing antibodies (bNAbs). This method makes use of the natural amino acid sequence virus variation that occurs in each HIV-infected individual to establish panels of closely related envelope proteins that differ from each other by a limited number of amino acid substitutions. We have previously used this method to identify a novel amino acid substitution in gp41 that conferred sensitivity to neutralization by monoclonal and polyclonal antibodies as well as virus entry inhibitors. In this paper, we describe a mutation in the V2 domain of gp120 that similarly induces a neutralization-sensitive phenotype in an otherwise neutralization-resistant envelope sequence.

Previous studies (10, 14, 33, 40, 43, 52, 72, 74) have suggested that sequences in the V2 domain act as the "global regulator of neutralization sensitivity" and confer neutralization resistance by restricting access to epitopes located in the V3 domain, the CD4 binding site, and chemokine receptor binding sites through "conformational masking" of neutralizing epitopes. Deletion of the V2 domain markedly increases neutralization sensitivity (10, 57, 62, 74), and several envelope proteins with V2 domain deletions have been developed as candidate HIV-1 vaccines (5, 42, 61). In this paper, we show that a single substitution of asparagine (N) for aspartic acid (D) at position 179 in the C-terminal portion of the V2 domain (corresponding to position 180 in HXB2 numbering) converts a highly neutralization-resistant virus to a neutralization-sensitive virus with a phenotype similar to that described for V2 domain deletion mutants. Position 179 has recently attracted attention as a critical element of the α4β7 integrin binding site that affects virus tropism to the gut (2). Our results suggest that mutation at position 179 results in a conformational change that increases neutralization sensitivity by exposure of epitopes in both gp120 and gp41 that are normally masked in the tri-

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meric structure of gp160 and thus are unavailable for antibody binding.

MATERIALS AND METHODS

Envelope genes and swarm analysis. Libraries of full-length envelope genes were isolated by reverse transcription-PCR (RT-PCR) from cryopreserved plasma samples from patients who became infected with HIV-1 while participating in the VAX004 phase 3 trial of the AIDSVAX B/B vaccine (20). The specimens selected for analysis represented recent infections with a mean estimated time after infection of 109 ± 58 days (48). A panel of clade B reference isolates was obtained from the NIH AIDS Reagent Repository and included JRCSF, YU2, QHO69.42, and TRO-11 (GenBank accession numbers U63632, M93258.1, AY835439, and AY835445). The JRCSF and YU2 envelope genes were isolated from proviral clones by PCR and cloned into an expression vector. The QH0692.42 and TRO-11 envelopes were obtained as full-length *Env*/*Rev* cassettes and were subcloned directly into the standard Monogram Biosciences expression vector for pseudovirus production. The swarm analysis protocol was described previously and is an application of the clonal analysis procedure developed by Monogram Biosciences (South San Francisco, CA) (19, 21, 22, 24, 28–32, 47). Briefly, the population of viral envelope genes present in the patient plasma was amplified by RT-PCR and then cloned into expression vectors. To test individual clones derived from the envelope population, the DNA was diluted and retransformed in bacteria, and individual clones were selected and screened for infectivity using the Monogram Biosciences coreceptor tropism assay. Pseudotype viruses containing cloned envelope genes were prepared from each patient plasma sample in 293 HEK cells. Viruses from individual clones were screened for infectivity and chemokine receptor tropism in U87 cells transfected with CD4 and the CCR5 or CXCR4 chemokine receptors as described previously (69). Ten to 12 envelopes with high infectivity were selected from each individual and evaluated in virus neutralization assays (described below).

***In vitro* mutagenesis.** Mutations were introduced into HIV-1 envelope proteins by site-directed mutagenesis using a QuikChange Lightning kit (Agilent, Santa Clara, CA) followed by confirmatory sequencing. Chimeric envelope genes were created by transferring PCR-amplified fragments between neutralization-sensitive and -resistant mutants. To facilitate this transfer, novel restriction sites preserving the virus sequence were introduced.

Antibodies and antiviral drugs. Four sera (Z23, Z1679, Z1684, and N16) from HIV-1 infected individuals (HIV-1-positive sera) known from previous studies (18, 59) to possess bNAbs were provided by Monogram Biosciences, Inc. (South San Francisco, CA). Six monoclonal antibodies (MAbs) with broadly neutralizing activity were obtained from the NIH AIDS Reagent Repository and/or Polymun AG (Vienna, Austria). These included 2G12, b12, 17b, 2F5, 4E10, and 447D-52 (4, 9, 15, 26, 46, 64, 66, 67, 77). MAbs to the α4β7 integrins were obtained from two sources. The Act-1 MAb (38) was obtained from the NIH AIDS Reagent Repository, and the α4/VLA-4/CD49d MAb was purchased from R&D Biosystems (Minneapolis, MN). A cyclized synthetic peptide (CWLDVC) reported to be a ligand for α4β7 (2) was obtained from GenScript (Piscataway, NJ). The antiviral compound CD4-IgG was described previously (3, 11) and provided by GSID (South San Francisco, CA). The peptide-based antiviral drug enfuvirtide (Fuzeon) was commercially available and produced by Roche, Inc. (Basel, Switzerland).

Virus neutralization assay. The study utilized a high-throughput virus neutralization assay to measure the ability of monoclonal antibodies and antibodies in HIV-1-positive plasma to inhibit infection of pseudotype viruses (17, 49, 55, 59). Briefly, pseudotype viruses were prepared by cotransfecting 293 cells with an envelope expression vector and an envelope-deficient HIV-1 genomic vector carrying a luciferase reporter gene. The virus-antibody mixture was incubated for 1 h prior to inoculation of U87 cells expressing CD4, CCR5, and CXCR4. Cells were then incubated for 3 days, and then viral infectivity was measured by luciferase expression. Neutralization data were reported as the 50% inhibitory concentration (IC_{50}) calculated from serum dilution curves. The positive controls included pseudoviruses prepared from the neutralization-sensitive HIV-1 isolate NL43 and the less neutralization-sensitive primary isolate JRCSF. The negative virus control consisted of pseudotype viruses prepared from the envelope of the amphotropic murine leukemia virus (aMLV). HIV-1 neutralization titers were considered significant only if they were greater than three times the aMLV titers.

Sequence analysis. The Los Alamos HIV database (http://hiv.lanl.gov/), the GSID HIV Data Browser (http://www.gsid.org/gsid_hiv_data_browser.html), and the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore) were interrogated to determine the degree of amino acid conservation at position 179 (180 according to HXB2 numbering). Alignments were performed using the EMBOSS suite (54). Because of insertions and deletions, it was not practical to identify each amino acid by use of the standard HXB2 numbering. Amino acid positions are provided with reference to the sequences from the envelope genes from clones 108051-005 and 108051-006 (GenBank accession numbers HM769943 and HM769944, respectively). Wherever possible, corresponding HXB2 numbering is provided in the text along with the 108051 numbering. An amino acid sequence alignment of the envelope proteins from clones 005 and 006 of the 108051 virus as well as the HXB2 envelope reference sequence is provided in Fig. S1 in the supplemental material.

TABLE 1. Neutralization of pseudoviruses containing HIV-1 envelope genes from subject 108051

Clone[a]	Neutralizing antibody titer (IC_{50}) for indicated human HIV-positive serum sample[b]			
	Z23	N16	Z1684	Z1679
005	<40	<40	<40	<40
006	**1,114**	**354**	**490**	**824**
009	<40	<40	<40	<40
011	<40	<40	<40	<40
013	<40	<40	<40	<40
015	96	**164**	**87**	**172**
016	<40	<40	<40	<40
018	42	73	<40	56
021	<40	<40	<40	<40
022	45	50	43	72

[a] "Clone" indicates the pseudotype virus prepared using the specified gp160 envelope genes. All clones tested were CCR5 tropic.

[b] HIV-positive sera Z23, N16, Z1684, and Z1679, known to possess broadly neutralizing antibodies (bNAbs). The neutralizing antibody titer (IC_{50}) is defined as the reciprocal of the plasma dilution that produces a 50% inhibition in target cell infection. Values in bold represent significant neutralization titers that are at least 3 times greater than those observed for the negative-control virus (aMLV).

RESULTS

In previous studies (47), we described the analysis of clade B envelope genes obtained from a cohort of 28 individuals infected with HIV-1 during the course of the VAX004 HIV vaccine trial that ran from 1998 to 2003 (20). In these studies, we identified seven cases where neutralization-sensitive and neutralization-resistant clones were both observed in the same individual. The first pair of envelopes analyzed was obtained from subject 108060 and allowed us to identify a mutation in a previously unexplored hydrogen-bonded ring structure that conferred sensitivity and resistance to bNAbs. In this paper, we report the analysis of viruses obtained from another individual (108051) in this cohort. The envelope genes from subject 108051 were amplified by RT-PCR from cryopreserved plasma collected at the first postdiagnosis blood draw. Envelope genes were analyzed for infectivity and chemokine receptor usage, and 10 envelopes with robust infectivity were isolated and evaluated in virus neutralization assays against a panel of HIV-1-positive sera, Z23, Z1679, Z1684, and N16, known to possess bNAbs (59). As can be seen in Table 1, most of the clones from patient 108051 were highly resistant to neutralization by all four sera, with only 2 of 10 (clones 006 and 015) being sensitive to neutralization. Based on the magnitude of the difference in neutralization titers, we selected clones 005 and 006 for further studies. Clone 015 gave a somewhat different pattern of neutralization sensitivity and was set aside for future studies. When we sequenced and aligned the translated gene products, we found a total of 25 individual amino acid differences between the sensitive and resistant clones (Fig. 1A). Some differences were due to isolated amino acid substitutions, and

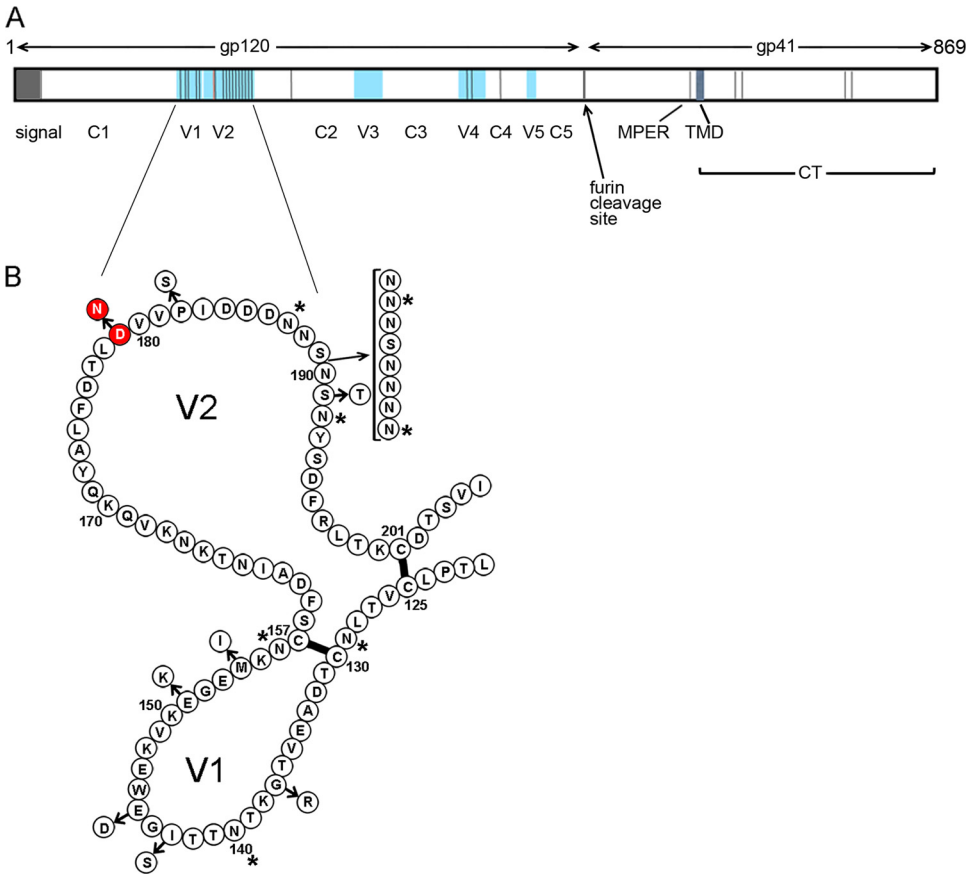

FIG. 1. Location of amino acid differences between neutralization-sensitive and -resistant clones isolated from subject 108051. HIV-1 envelope genes were isolated from the swarm of variants in plasma from subject 108051 and tested for sensitivity and resistance to neutralization. (A) The sequences of the neutralization-resistant clone 005 and the neutralization-sensitive clone 006 were aligned, and amino acid differences (vertical lines) were located on the linear sequence. Conserved (C) and variable (V) domains (blue) of gp120 are indicated, as well as the locations of the signal sequence (signal), membrane-proximal external domain (MPER), transmembrane domain (TMD), and cytoplasmic tail (CT). (B) Amino acid sequence differences in the V1 and V2 domains between the neutralization-resistant clone 005 (contiguous sequence) and neutralization-sensitive clone 006 (circles with arrows). Red circles indicate the locations of the D179N mutation. Open circles indicate the positions of other amino acid substitutions. Asterisks indicate the positions of N-linked glycosylation sites.

others represented clusters of differences resulting from deletions and insertions. Further examination revealed that 16 of the 25 amino acid differences were located in the V1 and V2 domains (Fig. 1B). To localize the amino acids responsible for the difference in neutralization sensitivity, we systematically transferred sequences individually and in clusters from the sensitive clone 006 envelope protein into the resistant clone 005 protein.

Identification of a mutation in gp160 from subject 108051 that confers sensitivity to neutralization by HIV-1-positive sera. When the panel of mutants was examined (Table 2), we found that single-amino-acid substitutions at positions 272, 462, and 644 had no effect on neutralization sensitivity or resistance. Similarly, a cluster of mutations in the cytoplasmic tail at positions 746, 748, 846, and 847 had no effect on sensitivity or resistance. Likewise, a cluster of amino acid substitutions at the C-terminal portion of gp120, including substitutions at positions 412, 413, and 462, had no effect on sensitivity. However, we found that the chimeric envelope protein containing the V1/V2 domain from clone 006 inserted into the backbone of the 005 envelope gene markedly increased sensitivity and exhibited neutralization titers comparable to those seen with the neutralization-sensitive clone 006.

These studies located the sequences responsible for increased neutralization sensitivity to either the V1 domain or the V2 domain. We then carried out further experiments to determine which domain was responsible (Table 2). We found that replacement of the clone 005 V1 domain with the V1 domain from clone 006 (V1_006) did not confer increased neutralization sensitivity. However, replacement of the V2 domain of clone 005 with that from clone 006 resulted in increased sensitivity, similar to that of the neutralization-sensitive clone 006 (Table 2). In the converse experiment, we transferred the V1 and V2 sequences of the resistant clone 005 envelope protein into the sensitive clone 006 envelope protein. Transferring the V1 domain preserved the neutralization-sensitive phenotype, whereas transferring the V2 domain resulted in loss of the neutralization-sensitive phenotype. Together, these studies clearly indicated that the difference in neutralization sensitivity between clones 005 and 006 could be attributed to the differences in the V2 domain.

Further studies were carried out to determine whether the

TABLE 2. Neutralization of pseudotype viruses with wild-type and mutated envelope genes from subject 108051 by HIV-positive sera possessing broadly neutralizing antibodies

Clone[a]	Mutation(s)	Neutralization antibody titer (IC_{50}) for indicated serum sample[b]			
		Z23	N16	Z1684	Z1679
005	wtR	<100	<20	<20	<20
006	wtS	**1,805**	**609**	**1,023**	**2,194**
005	T746I, K748E, V846R, R847Q	<100	<20	<20	<20
005	Y412Δ, T413S,G462D	<100	<20	<20	<20
005	E272K	<100	27	<20	<20
005	G462D	<100	<20	<20	<20
005	Q644R	<100	<20	<20	26
005	V1/V2_006	**2,123**	**749**	**1,328**	**2,177**
005	V1_006	<100	<20	<20	<20
005	V2_006	**1,457**	**390**	**827**	**1,412**
006	V1_005	**1,930**	**827**	**763**	**2,786**
006	V2_005	<100	<20	<20	<20
005	189 insert NNNSNNN, S191T	<100	<20	<20	<20
005	P182S	<100	<20	<20	<20
005	D179N	**1,875**	**272**	**1,024**	**3,094**
005	D179N, P182S	**1,391**	**773**	**584**	**2,731**
006	N179D	<100	<20	<20	<20
006	N179D, S182P	<100	<20	<20	<20

[a] Wild-type resistant (wtR) and wild-type sensitive (wtS) clones from subject 108051 are indicated. The V1_006 and V2_006 designations indicate chimeric envelope proteins in which the V1 and/or V2 domain of clone 006 replaces that of clone 005. V1_005 and V2_005 designations indicate chimeric envelope proteins in which the V1 and/or V2 domain of clone 005 replaces that of clone 006. "Δ" indicates deletion.

[b] The neutralizing antibody titer (IC_{50}) is defined as the reciprocal of the plasma dilution that produces a 50% inhibition in target cell infection. Values in bold represent neutralization titers that are at least 3 times greater than those observed for the negative control (aMLV). All clones tested were CCR5 tropic.

increase in neutralization sensitivity could be localized to specific amino acid substitutions in the V2 domain. As described above, there were 11 amino acid differences in the V2 domain between the sensitive and resistant clones of the 108051 virus. Further mutagenesis enabled us to rule out an eight-amino-acid insertion between positions 189 and 190 as well as single-amino-acid changes at positions 182 and 191 (Table 2). However, the single-amino-acid substitution of asparagine (N) for aspartic acid (D) at position 179 (corresponding to HXB2 position 180) markedly increased neutralization sensitivity and clearly accounted for the difference in neutralization between the neutralization-resistant clone 005 and the neutralization-sensitive clone 006.

In these studies, it can be seen that the largest increases in neutralization sensitivity occurred with the Z1679 and Z1684 sera, where neutralization sensitivity increased by 150-fold and 50-fold, respectively, compared to the level for the neutralization-resistant clone 005. The effect on sensitivity to N16 and Z23 was more moderate, with 13- and 18-fold increases, respectively, possibly indicating some differences in the magnitude and/or specificity of particular neutralizing antibody populations in each of the sera. The reverse mutation of N to D at position 179 conferred neutralization resistance to the neutralization-sensitive 006 clone, unambiguously confirming the importance of D at position 179 in conferring the sensitive phenotype. The fact that multiple single-amino-acid substitutions or clusters of substitutions at other locations within gp160 had no effect on the neutralization phenotype showed that increased neutralization sensitivity is not a trivial artifact. Rather, only specific amino acid substitutions at specific sites are able to convert a neutralization-resistant virus to a neutralization-sensitive virus (Tables 2 and 3).

Sensitivity to neutralization by MAbs and virus entry inhibitors. In order to investigate the mechanism by which the

TABLE 3. Sensitivity of 10851 mutants to neutralizing monoclonal antibodies and entry inhibitors

Clone[a]	Mutation(s)	IC_{50} (μg/ml) of indicated MAb or fusion inhibitor[b]					
		CD4-IgG	2F5	4E10	447-D52	17b	Enfuvirtide
005	wtR	>20	>20	>20	>20	>20	**0.308**
006	wtS	**0.14**	**1.29**	**0.21**	>20	**13.34**	**0.020**
005	D179N	**0.10**	**0.96**	**0.15**	>20	**4.25**	**0.023**
006	N179D	>20	**16.29**	**8.02**	>20	>20	**0.178**
005	D179E	**0.18**	**1.74**	**0.59**	>20	**12.11**	**0.091**
005	D179Q	**0.17**	**0.43**	**0.14**	>20	**2.64**	**0.036**
005	D179H	**0.11**	**0.55**	**0.11**	>20	**3.29**	**0.032**
005	D179S	**0.15**	**0.53**	**0.15**	>20	**5.60**	**0.037**
005	D179A	**0.09**	**0.66**	**0.10**	>20	**4.35**	**0.037**
005	P182S	>20	>20	>20	ND	ND	**0.182**
005	D179N, P182S	**0.221**	**1.314**	**1.585**	ND	ND	**0.018**
005	189 insert NNNSNNN, S191T	>20	>20	>20	ND	ND	**0.096**
005	V1/V2_006 sensitive	**0.097**	**0.935**	**1.117**	ND	ND	**0.018**
005	V1_006 sensitive	>20	19.04	>20	ND	ND	**0.173**
005	V2_006 sensitive	**0.357**	**1.670**	**2.135**	ND	ND	**0.017**
006	V1_005 resistant	**0.146**	**0.641**	**0.965**	ND	ND	**0.010**
006	V2_005 resistant	>20	>20	>20	ND	ND	**0.096**

[a] wtR, wild-type resistant clone; wtS, wild-type sensitive clone; ND, not done. The V1_006 and V2_006 designations indicate chimeric envelope proteins in which the V1 and/or V2 domain of clone 006 replaces that of clone 005. The V1_005 and V2_005 designations indicate chimeric envelope proteins in which the V1 and/or V2 domain of clone 005 replaces that of clone 006. All clones tested were CCR5 tropic. The IC_{50}s for b12 and 2G12 for all clones and mutations tested were >20 μg/ml.

[b] The neutralizing antibody titer (IC_{50}) is defined as the concentration of monoclonal antibodies or antiviral entry inhibitor that produces a 50% inhibition in target cell infection. IC_{50} values in bold print are at least 3 times greater than the IC_{50} values measured for the specificity control virus (aMLV) and are therefore considered positive for neutralization in this assay.

replacement of D with N at position 179 in the V2 loop alters neutralization sensitivity, we investigated the effects of monoclonal antibodies (MAbs) and virus entry inhibitors that target defined regions of the envelope protein (Table 3). These included the b12, 17b, 2G12, and 447D-52 MAbs, known to neutralize HIV-1 and bind to epitopes in gp120 (4, 9, 15, 26, 66, 67), and the 2F5 and 4E10 MAbs, known to bind to epitopes in gp41 (46, 64, 77). In addition to these MAbs, we also made use of the antiviral entry inhibitors CD4-IgG and enfuvirtide to further define the mechanism of neutralization sensitivity. The antiviral entry inhibitor CD4-IgG binds to the CD4 binding site in gp120 and is able to neutralize laboratory-adapted CXCR4-dependent isolates at low concentration (0.01 to 0.1 μg/ml) and primary CCR5-dependent primary isolates of HIV-1 (16) at a high concentration (10 to 100 μg/ml). Enfuvirtide is a peptide-based virus entry inhibitor (39, 70) that is thought to interfere with the formation of the six-helix bundle that is required for virus fusion. It is thought to bind to the prehairpin intermediate structure of gp41 that is transiently formed in gp41 (45) after binding of CD4 to the gp160 trimer (12, 23, 36).

The results obtained with this panel of inhibitors are shown in Table 3. We found that the wild-type neutralization-resistant clone 005 was resistant to all of the MAbs in the panel as well as to CD4-IgG. Because of its resistance to four HIV-1-positive sera possessing bNAbs, as well as its resistance to the broadly neutralizing MAbs 2F5, 4E10, 2G12, b12, and 447-D and to CD4-IgG, clone 005 appears to be a tier 3 (44) neutralization-resistant virus (D. Montefiori, personal communication). When we examined the properties of clone 006, we found that it was also completely resistant to neutralization by the b12, 2G12, and 447D-52 antibodies. Examination of the amino acid sequences of clones 005 and 006 showed that both envelopes contained polymorphisms in the epitopes recognized by these three MAbs. The 108051 envelope proteins all possess a GPGG sequence at the tip of the V3 loop rather than the clade B consensus GPGR motif required for 447D-52 binding (15, 26, 63). Similarly, the 108051 residues T286, T375, and M376 are known to be common in b12-resistant viruses and differed from the A281, V372, and T373 (HXB2 numbering) residues, common in b12-sensitive viruses (71, 75). Finally, the lack of inhibition by the 2G12 antibody could be attributed to the fact that the 108051 envelope protein lacks two of four glycosylation sites at positions 300 and 395 (corresponding to HXB2 positions 295 and 392) essential for binding by this antibody (58). Thus, the resistance of clones 005 and 006 to the b12, 2G12, and 447D-52 antibodies could be attributed to polymorphisms at neutralizing epitopes. However, since antibodies with specificities similar to those of b12 and 2G12 are rare in HIV-1-positive sera (8, 56), another explanation was required to account for the neutralization resistance of clone 005 to polyclonal sera (Table 1) and the remaining monoclonal antibodies in this panel (Table 3).

First, we examined the sensitivity of the neutralization-sensitive and -resistant variants to CD4-IgG (Table 3). Previous studies (36, 37, 41) have shown that the CD4 binding site is located entirely within the gp120 portion of the HIV-1 envelope protein, recessed deeply below the apex in the native trimer. We found that a high CD4-IgG concentration (>20 μg/ml) was required for neutralization of 108051 clone 005, which was consistent with the concentration required to neutralize other primary isolates of CCR5-dependent viruses (16). Replacement of D with N at position 179 in clone 005 increased sensitivity to CD4-IgG approximately 200 times compared to the level for the wild-type neutralization-resistant clone 005 envelope. Conversely, we found that the neutralization-sensitive clone 006 could be converted to the CD4-IgG-resistant phenotype by replacement of N with D at position 179.

We next examined the effect of the D179N mutation on 17b, a neutralizing MAb known to target a conserved CD4-induced (CD4i) epitope on gp120 overlapping the coreceptor binding region (36, 66, 67). Clone 005 was resistant to neutralization by 17b at 20 μg/ml, and the neutralization-sensitive clone 006 was marginally more sensitive, with an IC_{50} of 13.3 μg/ml (Table 3). However, the clone 005 envelope with the D179N mutation was approximately 5-fold more sensitive to neutralization by this antibody. This result suggests that the D179N mutation enhances neutralization by the 17b MAb but suggests that other sequence differences between clone 005 and clone 006 also affect the binding of this antibody.

We next considered the effect of the D179N mutation on sensitivity to MAbs and entry inhibitors that target sites in the gp41 protein (Table 3). Interestingly, replacement of D with N at position 179 in the V2 domain had a significant effect on sensitivity to neutralization by the two broadly neutralizing MAbs 4E10 and 2F5, directed to the membrane-proximal external region (MPER) of gp41. The epitopes recognized by these antibodies are well defined, with the 2F5 MAb recognizing the ELDWA sequence and the 4E10 MAb recognizing the adjacent NWF(D/N)IT sequence (46, 76, 77). Recent studies suggest that the peptide in which these sequences occur is partially embedded in the lipid bilayer (60). We found that the D179N mutation increased neutralization sensitivity approximately 20-fold in the case of 2F5 and between 100- and 200-fold in the case of 4E10. This result showed that a single-amino-acid substitution in gp120 could have a dramatic effect on the neutralizing activity of antibodies directed to the gp41 domain. Similar results were obtained with the antiviral entry inhibitor enfuvirtide. This drug consists of a peptide derived from gp41 sequences that overlap the MPER domain and the C34 helix (70). The binding of enfuvirtide to gp41 is thought to depend on CD4 binding which induces a conformational change that exposes a binding site involving the HR1 domain of gp41 (25). The observation that sensitivity to enfuvirtide was increased 13-fold (Table 3) in the D179N mutant provides additional evidence that a mutation in the V2 domain of gp120 can modulate the potency of antiviral compounds targeting the gp41-mediated virus fusion mechanism.

Conservation of aspartic acid at position 179. Comparative sequence analysis showed that position 179 (corresponding to HXB2 position 180) is highly conserved across all clades of HIV-1. We analyzed 5,918 sequences from 2,414 individuals in three datasets, including 1,963 curated and aligned sequences from the Los Alamos HIV Sequence database (HIV-1/SIVcpz; 2008) that listed one sequence per individual, a set of 2,908 sequences from 102 individuals with acute infections (1, 34), and 1,047 sequences from 349 individuals with recent infections from the VAX004 HIV vaccine trial (GSID HIV Data Browser [http://www.gsid.org/gsid_hiv_data_browser.html]). We found only a single naturally occurring HIV-1 sequence,

TABLE 4. Envelope protein mutagenesis for investigation of the significance of aspartic acid at position 179 in clones of the 108051 and 108048 envelope proteins

Clone[a]	Mutation(s)	Neutralization antibody titer (IC_{50}) for indicated serum sample[b]			
		Z23	N16	Z1684	Z1679
005	wtR	<100	<40	<40	<40
006	wtS	**1,524**	**276**	**832**	**705**
005	D179N	**3,963**	**662**	**873**	**602**
006	N179D	<100	<40	<40	<40
005	D179E	**1,792**	**167**	**611**	**261**
005	D179Q	**4,997**	**524**	**1,661**	**1,020**
005	D179H	**5,121**	**623**	**1,078**	**1,410**
005	D179S	**3,626**	**379**	**859**	**816**
005	D179A	**3,804**	**503**	**1,056**	**1,137**
005	L178R, D179G, V180D	**3,001**	**289**	**563**	**921**
108048_002	wtR	**119**	**55**	<40	<40
108048_002	D179N	NI	NI	NI	NI
108048_002	V1/V2_006	**12,541**	**1,729**	**2,729**	**1,528**

[a] Neutralization-resistant and -sensitive clones were obtained from subject 108051 (clones 005 and 006) or from subject 108048. V1/V2_006 represents a chimeric envelope where the V1 and V2 domains of 108051_006 replaced the V1 and V2 domains of the 108048_002 envelope. "NI" indicates no infectivity.

[b] The neutralizing antibody titer (IC_{50}) is defined as the reciprocal of the plasma dilution that produces a 50% inhibition in target cell infection. Values in bold represent neutralization titers that are at least 3 times greater than those observed for the negative control (aMLV). All clones tested were CCR5 tropic.

other than the 108051 sequence from the GSID HIV Sequence database, where N replaced D at position 179 (GenBank accession number AF321080). Interestingly, we also found that the D in the LDI/LDV motif was conserved in simian immunodeficiency virus (SIV) and HIV-2, where it corresponded to position 201 in the SIV reference sequence (GenBank accession number M33262). We found that this residue was conserved in all 69 different HIV-2 and SIV sequences in the HIV-2/SIV/MN 2008 Los Alamos HIV Sequence database. Given the high degree of sequence variation among these primate lentiviruses, D179 would be preserved over time and across species only if it played an important role in the survival of these viruses.

Further studies were carried out to try to understand the mechanism by which aspartic acid at position 179 modulates neutralization sensitivity in 108051. In these studies, we constructed a series of mutants where D at position 179 was replaced by other amino acids (Tables 3 and 4). We found that replacement of D at 179 with the hydrophilic, basic amino acids arginine (R) and lysine (K) or the hydrophobic branched-chain isoleucine (I) residue failed to yield infectious virus. This result suggests that D179 must interact with other parts of the envelope protein and that these interactions can alter virus infectivity. In contrast, it was possible to replace D179 with other amino acids that preserved virus infectivity. For example, replacement of D with amino acids with short side chains, such as alanine (A), serine (S), and glycine (G), resulted in infectious viruses. Replacement of D179 with bulky side chains, such as histidine (H), glutamine (Q), or the negatively charged glutamic acid (E), also resulted in infectious viruses. However, all of these replacements increased sensitivity to neutralization by the polyclonal HIV-positive sera (Table 4) and the 2F5, 4E10, and 17b MAbs as well as CD4-IgG and enfuvirtide (Table 3). Replacement of D with glutamic acid (E), whose acidic side chain is only 1 carbon longer than D, preserved CCR5 tropism but similarly increased neutralization sensitivity. This result indicates that there must be an extremely restrictive structural constraint required to preserve neutralization resistance. Thus, the only amino acid that we have found that can maintain the neutralization-resistant phenotype is D at position 179.

In theory, the high level of conservation of D179 might be critical for maintaining the conformation of the envelope protein or might be involved with receptor binding. Indeed, D179 has recently been highlighted as part of the LDV/I recognition motif that forms the newly described $\alpha4\beta7$ receptor binding site on gp120 (2). Based on this observation, we examined the effect of $\alpha4\beta7$ binding inhibitors on virus neutralization in order to determine if disruption of $\alpha4\beta7$ binding could account for the observed increase in neutralization sensitivity associated with the D179N mutation. We found (see Table S1 in the supplemental material) that neither the Act-1 MAb to $\alpha4\beta7$ nor the cyclic peptide inhibitor CWLDVC (2) was able to inhibit the infectivity of JRCSF, NL43, or the wild-type neutralization-sensitive and -resistant clones of 108051 in the U87 cell pseudotype neutralization assay. However, both inhibitors (Act-1 and cyclic CWLDVC) were able to prevent the binding of recombinant gp120 to a cell line (65) expressing $\alpha4\beta7$ in a flow cytometry assay (D. Fonseca and P. Berman, unpublished results). These results suggest that the U87 target cells used in our assay lack the $\alpha4\beta7$ receptor and demonstrate that the increased neutralization sensitivity of the D179N mutant cannot be attributed to disruption of interactions mediated by $\alpha4\beta7$ in our assay system. However, this mutation might be expected to interfere with infectivity in systems where the $\alpha4\beta7$ receptor is expressed on target cells.

Transfer of the D179N mutation to other viruses. To investigate possible strain-specific differences of the D179N mutation on increased neutralization sensitivity, we attempted to transfer this mutation to five other, unrelated viruses. For this purpose, we selected four commonly used tier 2 viruses from standard neutralization panels exhibiting a range of neutralization sensitivities, specifically JRCSF, YU2, QH0692, and TRO-11. In addition, we also examined neutralization sensitivity in another virus, 108048, from the VAX004 trial. All five viruses possessed D at the position corresponding to position 179 of the 108051 virus. Whereas all five wild-type viruses were infectious in the U87 pseudotype assay, we found that replacement of D with N at positions corresponding to 108051 position 179 resulted in viruses with little or no infectivity. This result suggested that D179N is essential for infectivity and suggests that compensatory mutations may be necessary to preserve infectivity when D is replaced by N at this position. To further explore this possibility, we replaced the entire V1 and V2 domains of the neutralization-resistant 108048 virus with that of the 108051 virus containing the D179N mutation. As can be seen in Table 4, replacement of the entire V1 and V2 domains from 108051 markedly increased sensitivity to neutralization by the 4 HIV-1-positive sera, suggesting that the compensatory mutations required to increase neutralization sensitivity while preserving infectivity are located within the V1 or V2 domains.

DISCUSSION

The results presented in this study show that a single-amino-acid mutation, D179N, in the V2 domain of gp120 can convert a highly neutralization-resistant virus to a neutralization-sensitive virus. The fact that the D179N mutation increased sensitivity to neutralization by MAbs and antiviral drugs, targeting both gp120 and gp41, suggests that the D179N mutation induces a conformation change that affects accessibility of multiple neutralizing epitopes, rather than affecting the contact residues of a single neutralizing antibody binding site. These results suggest a far greater level of interaction between these two subunits, with respect to neutralization sensitivity, than was previously appreciated. The fact that D179 is conserved in HIV-1, SIV, and HIV-2 suggests that D at position 179 may have been preserved throughout evolution in order to preserve resistance to neutralization by antibodies targeting epitopes in both gp120 and gp41.

Our results are consistent with previous studies that have identified the V2 domain of gp120 as the "global regulator of neutralization sensitivity" (51, 52). Because the V2 domain can be deleted entirely in some viruses while preserving virus viability (10, 57, 62), it seems unlikely that the V2 domain provides a contact surface required for infectivity or virus fusion. Rather, it appears to provide an epitope-"masking" function that is thought to conceal important neutralizing epitopes from neutralizing antibodies until the envelope protein undergoes a conformational change triggered by CD4 binding (35, 36, 41). This hypothesis is supported by studies showing increased binding of antibodies to neutralizing epitopes in the V3 and C4 domains by envelope proteins lacking the V2 domain (10, 50, 52, 62, 72). In this regard, the single-amino-acid substitution of N for D at position 179 appears to confer the same phenotype as that observed when the entire V2 domain is deleted from the SF162 virus (5, 10, 27, 61, 62, 72–74). Further data supporting the role of the V2 domain in regulating neutralization sensitivity is provided by studies showing that sensitivity and resistance to neutralization can be transferred by moving the V2 domain from a neutralization-sensitive virus (e.g., SF162) onto a neutralization-resistant virus (e.g., JR-FL) backbone. Conversely, the neutralization-sensitive SF162 virus can be converted to a neutralization-resistant virus by exchange of the V2 domain with that of JR-FL (52).

Although conformational masking by the V2 domain appears to explain most of the data relating to the ability of the V2 domain to modulate neutralization sensitivity and resistance (10, 43, 50, 52, 62, 72, 74), the molecular interactions determining how the mask is "raised and lowered" have not been characterized. Our results suggest that D179 mediates a key interaction required for maintenance of the neutralization-resistant, "masked" state. Replacement of D with N at position 179 seems to open up the structure of the gp160 trimer and makes the virus more sensitive to neutralization by exposing epitopes in both gp120 and gp41. Aspartic acid at position 179 appears to be unique, since it appears in all but two of more than 5,918 virus sequences in the 3 datasets examined and since all of the other mutations created *in vitro* at this position resulted in either noninfectious viruses or viruses with increased neutralization sensitivity. The lack of representation of viruses with mutations at position 179 in other data sets might reflect the fact that all other variants are noninfectious or are so sensitive to neutralization that they are rapidly eliminated from circulation once envelope-specific antibody responses have developed. The fact that transfer of the D179N mutation to five unrelated viruses (YU2, JRCSF, QH0692.42, TRO-11, and 108048) all resulted in noninfectious viruses is consistent with the importance of D179 in preserving the functional structure of the envelope protein and suggests that compensatory mutations are required in other parts of the molecule to preserve infectivity when D179 is replaced with N. In this regard, the need for compensatory mutations may be similar to that observed with V2 domain deletions where deletion of the V2 domain in the SF162 strain results in infectious viruses, whereas deletion of the V2 domain in other strains (e.g., HXB2) requires compensatory mutations to maintain virus infectivity (57). This possibility is supported by the V1/V2 domain replacement experiment (Table 4), where it was found that replacement of the entire V1/V2 domain could increase sensitivity to neutralization by HIV-1-positive sera, while preserving infectivity. With respect to mutations at position 179, the amino acid substitutions that destroyed infectivity may have stabilized the masking function to such an extent as to prevent the conformational changes required for infectivity following receptor binding.

Our data are also consistent with the hypothesis that the V2 masking function is dependent on quaternary interactions between the gp160 subunits that associate to form the trimeric envelope structure that mediates virus infectivity and fusion (13, 35, 36). Based on structural studies involving cryoelectron tomography and X-ray data fitting, the V1 and V2 domains appear to be located at the apex of an intermolecular contact region within the envelope glycoprotein trimer (41). According to this model, the native trimer is held together by strong contacts at the gp41 base and the V1/V2 regions, with little or no contact elsewhere. Upon CD4 binding, the monomers rotate with respect to the core of the trimer to "open" the center of the trimer, exposing CCR5 binding sites, shifting gp41 up toward the cell membrane to form the six-helix bundle, and exposing the fusion peptide at the target cell membrane (see Fig. S2 in the supplemental material). When viewed in the context of these observations, our data are consistent with the possibility that D179 provides interactions required to maintain the unligated trimeric structure. Accordingly, mutations at position 179 may weaken the quaternary, intersubunit interactions, thereby providing increased access of antibodies to parts of the molecule, such as the V3 domain, the CD4 binding site, and the MPER, that are normally located in the interior of the molecule and exposed only after CD4 binding. Further investigations using conformation-dependent antibodies to the V2 domain, such as the newly described PG9 and PG16 antibodies (68), might provide additional support for this model; studies using these antibodies as well as cryoelectron tomography are planned to further investigate this mutation.

The results reported herein confirm and extend our previous studies, in which swarm analysis has proved useful in identifying single-amino-acid substitutions that appear to trigger conformational changes that expose or conceal epitopes recognized by bNAbs. Envelopes with exposed neutralizing epitopes may represent a source of immunogens potentially more effective in eliciting bNAbs than those previously tested. Envelope proteins with deleted V2 domains have been tested as

candidate HIV-1 vaccine antigens and were shown to elicit higher titers of neutralizing antibodies than wild-type proteins (5, 42, 61, 73). Studies are in progress to determine whether immunization with the D179N mutant described in these studies exhibits broader neutralizing activity, as seen with the V2-deleted envelope antigens.

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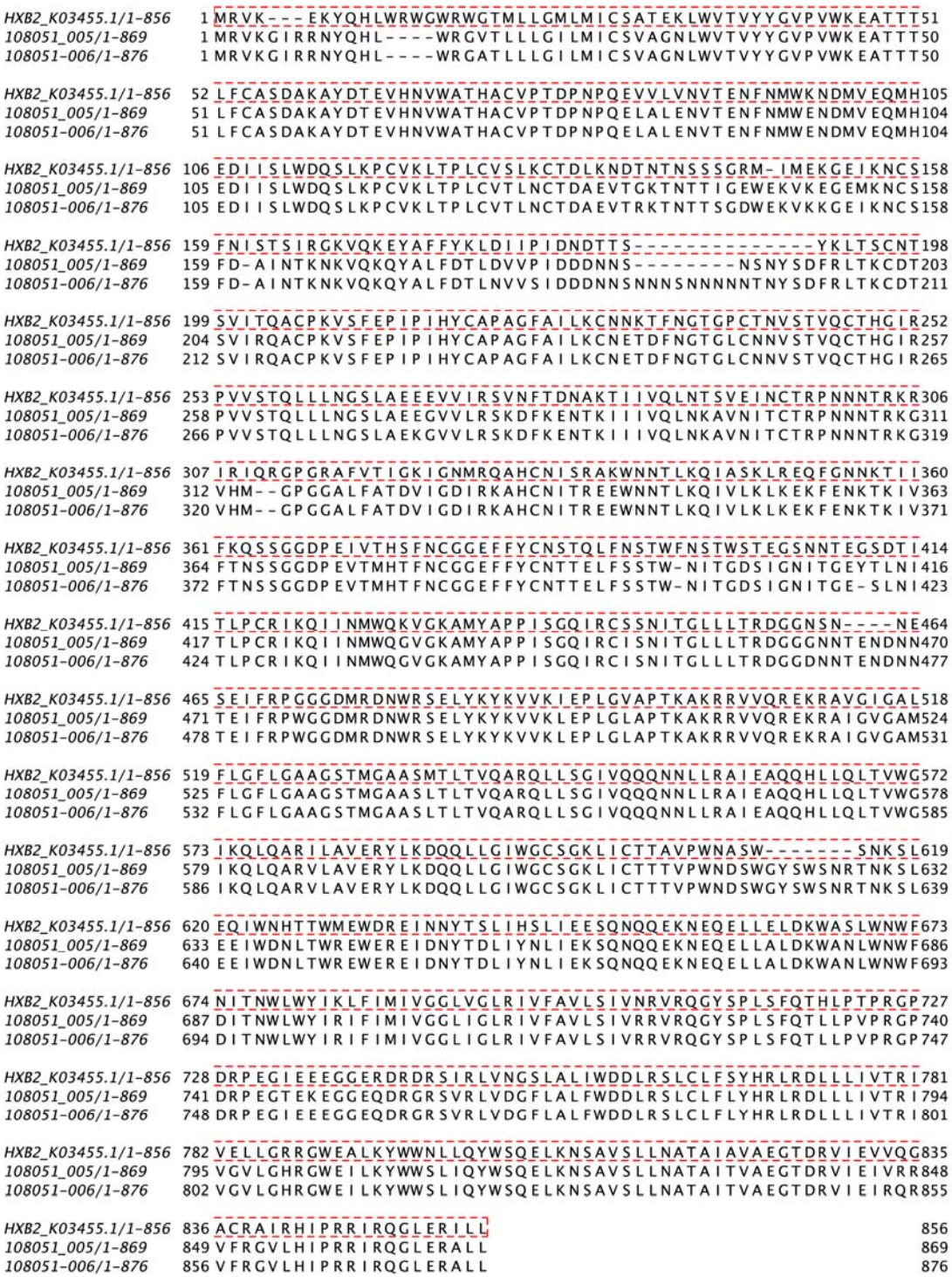

Figure S1. MAFFT alignment of HBX2 reference sequence and 108051 clones 005 and 006 (accession numbers K03455.1, HM769943 and HM769944 respectively).

Table S1. Effect of antibody and peptide inhibitors of α4β7 binding on the neutralization of viruses with envelopes from subject 108051

Viruses/ Clones [a]	IC50 (μg/mL antibody, μM peptide) of indicated MAb or fusion inhibitor [b]					
	Z23	Act-1 MAb	CWLDVC	Act-1 + CWLDVC	α4/CD49d MAb	α4/CD49d + CWLDVC
108051_005 wtR	<100	>20	14	>20	>20	>20
108051_006 wtS	**2298**	>20	12	>20	>20	>20
108051_005_D179N	**3758**	>20	12	>20	>20	>20
JRCSF	**422**	>20	19	>20	>20	>20
NL43	**3042**	>20	13	>20	>20	>20
aMLV	<100	>20	12	>20	>20	>20

[a] wtR, indicates wild type resistant; wtS, indicates wild type sensitive. MAbs Act-1 and CD49d are known to react with the α4β7and α4 chain of VLA4, respectively. CWLDVC is a synthetic peptide known to inhibit α4β7 binding. The CWLDVC peptide used at a 10μM concentration was known to inhibit gp120 binding to α4β7 in control experiments using the RPMI8866, α4β7-expressing cell line (65).

[b] Values in bold represent neutralization titers that are at least 3 times greater than those observed against the negative control (aMLV).

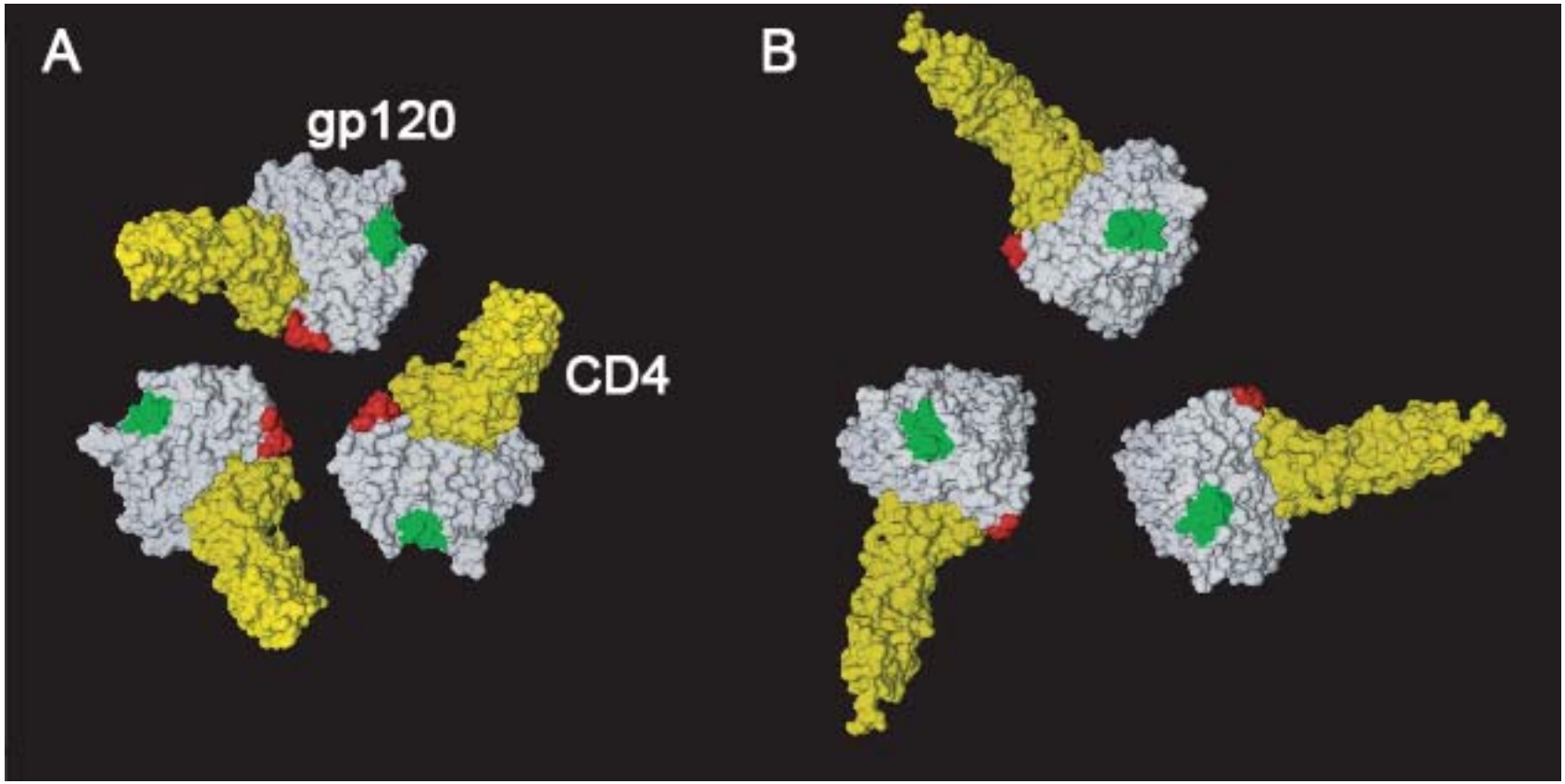

Figure S2. Conformational changes that affect the V2 domain in HIV envelope trimer before and after CD4 binding. Figure represents the orientation of gp120 monomers before (A) and after (B) a conformational change triggered by the binding of CD4. Gray, indicates gp120 monomers; yellow, indicates CD4; red, indicates the V2 domain stem; green, indicates the V3 domain stem. Following CD4 engagement, the monomers rotate and change position with respect to the central axis of symmetry. The distance between the V2 stems increases, and the V3 stems become positioned at the top. Data taken from Liu et al. (41).