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Length Polymorphism within the Second Variable Region of the Human Immunodeficiency Virus Type 1 Envelope Glycoprotein Affects Accessibility of the Receptor Binding Site

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Sequential mutations were introduced into the V2 region of human immunodeficiency virus (HIV) type 1 HXB2, affecting the length, charge, and number of potential glycosylation sites. The insertions had no effect on cytopathicity or on the ability of virus to replicate in peripheral blood mononuclear cells and established T-cell lines. However, deletion of amino acids 186 to 188, encoding a conserved glycosylation site, resulted in a nonviable virus, suggesting a minimal length requirement of 40 amino acids for a functional V2 loop. However, all amino acid insertions affected the sensitivity of the variants to neutralization by soluble CD4 and monoclonal antibodies specific for epitopes in the V3 and CD4 binding site regions. Furthermore, these mutant viruses showed resistance to neutralization by HIV-positive human sera. Soluble gp120 mutant glycoproteins showed increased affinities for soluble CD4 and monoclonal antibodies specific for a number of epitopes overlapping the CD4 binding site, confirming that length increases in V2 affect exposure of the CD4 binding site. In summary, these data demonstrate that differences in V2 length modulate immunoreactivity of the envelope glycoprotein and support an association between the V2 and CD4 binding site regions.

Human immunodeficiency virus type 1 (HIV-1) infects $CD4^+$ lymphocytes, monocytes, and dendritic cells in the peripheral blood and lymphoid organs (24, 33). This viral tropism correlates with expression of the cell surface antigen CD4, which has been shown to be the principal receptor interacting with the viral surface glycoprotein (gp) (16, 32). However, cell surface expression of CD4 alone is not sufficient to confer susceptibility to infection by HIV-1. Recently, several members of the chemokine receptor family of G protein-coupled seven-transmembrane-spanning proteins were identified as additional coreceptors (1, 9, 17–19, 20).

HIV-1 exhibits considerable sequence variability, much of which is seen in the envelope (*env*) gene. Variation in the envelope gp is clustered in five variable regions, V1 to V5, which are interspersed between five conserved regions, C1 to C5 (38, 41). These conserved regions are thought to constitute the functional core of the gp and include the minimal CD4 binding site (48). This variation results in a spectrum of viruses with differences in cell tropism, replication rate, and cytopathicity. HIV isolates may be categorized in vitro according to their ability to replicate in primary and established cell lines and to induce cytopathic effects. Some viruses are able to induce syncytia in their target cells (syncytium inducing [SI]) whereas others are not (non-syncytium inducing [NSI]) (4, 7, 30, 54, 55). Several reports have suggested that the appearance of SI viruses is associated with a more rapid CD4 cell decline and progression to disease (4, 7, 14, 22, 30, 50, 54, 55). Considerable effort has therefore focused on establishing which regions of the Env gp may be responsible for determining such phenotypes.

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The second and third variable regions (V2 and V3) have been reported as being the determinants of both tropism and cytopathicity, leading to the suggestion of signature patterns for prediction of viral phenotype (3, 8, 26, 27, 35, 51, 60). Furthermore, both of these regions have been reported to be targets for neutralizing antibodies, supporting a critical role for these regions in the virus-cell entry process. Groenink and colleagues (26) suggested that the configuration of a variable motif within V2 was predictive of an NSI-to-SI phenotypic switch, whereby an increase in length and positive charge was indicative of an SI phenotype. However, recent evidence suggests that the relationship between V2 length and NSI/SI phenotype is not as clearly defined as previously thought (2, 15, 47, 49, 56); for example, we demonstrated that V1-V2 regions amplified directly from infected peripheral blood mononuclear cells (PBMC) conferred NSI PBMC-tropic phenotypes to HXB2 (47).

At present no structural information for gp120 is available, such that protein-protein interactions are inferred from studies of viability and antigenicity of defined envelope mutants. To date, associations have been suggested between the V3 and CD4 binding site (44, 59, 64), V2 and V3 (2, 34), C1, C2, and C5 (45), and the V2 and C4 regions (23, 58). Moore and Sodroski (43) proposed a model for envelope topology based on monoclonal antibody (MAb) reactivities with LAI gp120, in which the V2 and V3 loops are exposed on the surface of the molecule. Given the reported interactions between the variable and conserved regions, it is possible that length polymorphism within the variable loops may affect env gp conformation.

The Env gp is highly glycosylated, and potential sites are located predominantly in the variable loops (38). Several reports suggest that these glycan modifications are essential for correct processing, folding, and oligomerization (21, 39, 62). It is interesting to note that deletions and/or insertions in the variable regions frequently encode repeated motifs containing

TABLE 1. Summary of V2 mutations[a]

Nomenclature	Mutation	Amino acid sequence	No. of glycosylation sites	Change in length	Change in charge
HXB2	wt	$_{185}$DNDTT$_{189}$	1	0	0
ΔA	Deletion of aa 186, 187, 188	$_{185}$DT$_{189}$		−3	+1
B0	186NS	$_{185}$DSDTT$_{189}$		0	0
B1	wt	$_{185}$DNDTT$_{189}$	1	0	0
C00	Addition of three residues between positions 185 and 189	$_{185}$DSDTSDTT$_{189}$	−	+3	−1
C10		$_{185}$DNDTSDTT$_{189}$	1	+3	−1
C01		$_{185}$DSDTNDTT$_{189}$	1	+3	−1
C11		$_{185}$DNDTNDTT$_{189}$	2	+3	−1
D100	Addition of six residues between positions 185 and 189	$_{185}$DNDTSDTSDTT$_{189}$	1	+6	−2
D010		$_{185}$DSDTNDTSDTT$_{189}$	1	+6	−2
D110		$_{185}$DNDTNDTSDTT$_{189}$	2	+6	−2
D111		$_{185}$DNDTNDTNDTT$_{189}$	3	+6	−2

[a] All mutagenesis was performed on a *Sal*I-*Bam*HI fragment of the molecular clone HXB2.MCS cloned into M13mp19 using the Kunkel method of site-directed mutagenesis. This had previously been mutated to contain unique *Hpa*I and *Ngo*MI sites, 5′ and 3′ of the V1-V2 region, respectively, enabling the replacement of novel V1-V2 sequences (47). Oligonucleotides were designed such that the base inserted at the R position (ART) encoded either N (AAT) or S (AGT), leading to the creation or deletion of potential glycosylation sites. The oligonucleotides also introduced a silent *Cla*I site to enable rapid screening of mutants. M13 plaques were screened by PCR for the presence of this *Cla*I site; clones which gave rise to *Cla*I-digestible PCR products were sequenced using the method of Sanger. PCR-amplified DNA was digested with *Hpa*I and *Ngo*MI and ligated to similarly digested pHXB2.MCS. Transformants were screened by PCR, and the presence of the *Cla*I site was confirmed.

potential glycosylation sites (5, 47, 56). We (47) and others (5, 15, 26, 36, 37, 56) have reported that sequence variation within V2 is most frequently associated with length changes in the carboxyl region. V2 sequences observed in vivo exhibit both substitutional and length polymorphism; it is therefore impossible to directly assess the biological significance of length change alone by analyzing such sequences. We therefore designed a series of mutants to assess the effects of change in V2 length, charge, and number of N-linked glycosylation sites on gp120 conformation and virus phenotype.

Replication of V2 mutant viruses. The changes introduced within the V2 region are summarized in Table 1 and affect overall length, charge, and predicted number of potential glycosylation sites, enabling us to systematically assess the role of such changes on viral phenotype. Transfection of B1 (wild type [wt]) and mutant plasmids into HeLa CD4 cells gave rise to both soluble (50 to 100 ng/ml) and intracellular p24 and gp120 antigens (data not shown). All viruses, with the exception of ΔA, resulted in multinucleated foci in the transfected cells, confirming that virus-mediated cell fusion had occurred (10). Titered stocks of wt and mutant viruses were allowed to infect the C8166 cell line at a multiplicity of infection of 0.01, and replication was monitored by extracellular p24 antigen production. All of the mutant viruses, with the exception of ΔA, had growth rates largely comparable to that of the wt virus (Fig. 1). Similar results were obtained for infection of PBMC and for the SupT1 and MT-2 T-cell lines (data not shown). The stability of the V2 insertions during virus replication was assessed by amplification of the V2 region from proviral DNA extracted from infected cultures. The amplified products from all cultures were found to be of the correct length and sequence and were stable over time (21 days) (data not shown).

To confirm that no additional changes had occurred in the ΔA envelope which may account for the nonviable nature of this clone, the entire open reading frame was sequenced and found to contain no additional changes (data not shown). Transfection of HeLa cells with the ΔA plasmid resulted in detectable levels of p24 and gp120 antigen, indicating that the transfection was successful; however, these viruses failed to replicate when passaged onto PBMC or T-cell lines (data not shown). We therefore conclude that deletion of amino acids 186 to 188 results in a nonviable virus. In order to elucidate the stage in the life cycle at which ΔA was blocked, we compared the ability of ΔA and wt viruses to enter HeLa-CD4 LTR-LacZ cells (11). Equivalent amounts of virion wt and ΔA p24 were allowed to infect the HeLa-CD4 LTR-LacZ cells. After 24 h the cells were washed and incubated with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and blue foci were counted. wt virus resulted in an average of 35 to 42 blue foci per well, indicating the presence of internalized virus. However ΔA failed to give rise to any blue cells, suggesting that ΔA virus is blocked at the level of entry. All foci observed were HIV specific since infection, and subsequent blue cell formation, could be neutralized by prior incubation of the virus with polyclonal human serum (data not shown). Detergent-solubilized ΔA viral gp120 was able to bind soluble CD4 (sCD4) with the same affinity as wt gp120, suggesting that the block to entry was post-CD4 receptor binding (data not shown). The predicted glycosylation site at residue 186 is well conserved, however, the adjacent carboxyl region sequences obtained directly from infected PBMC are often variable in length (47, 56). Disruption of this potential glycosylation site (mutant B0) resulted in a virus capable of replicating equivalently to wt, suggesting that the glycosylation site per se is not essential and its loss was not responsible for the nonviability of ΔA (37). This is in agreement with previous reports on the viability of various substitutional V2 mutants (53, 57, 58). It is interesting to note that the shortest V2 sequences obtained directly from infected PBMC were 40 amino acids, suggesting a minimum length for viability (47, 56).

Effect of V2 changes on gp120 conformation. In order to assess the effect(s) of V2 mutations on native envelope conformation, we cloned the gp120 open reading frames of B1, C10, and D100 into the expression vector pcDNA3. These mutants were selected because they enable the effect of length to be monitored while maintaining the position and number of potential glycosylation sites. Transient transfection resulted in soluble gp120 expression (1 to 2 μg/ml) which was quantified by a capture enzyme-linked immunosorbent assay (ELISA). Equivalent concentrations of the wt and mutant gp120 proteins were tested for their ability to bind sCD4 and a panel of MAbs specific for linear and conformation-dependent epitopes; concentrations of MAbs required to give half-maximal binding were calculated using a weighted Line-Weaver Burke algorithm (Table 2).

MAbs 10/76b and 11/4c, which recognize linear epitopes

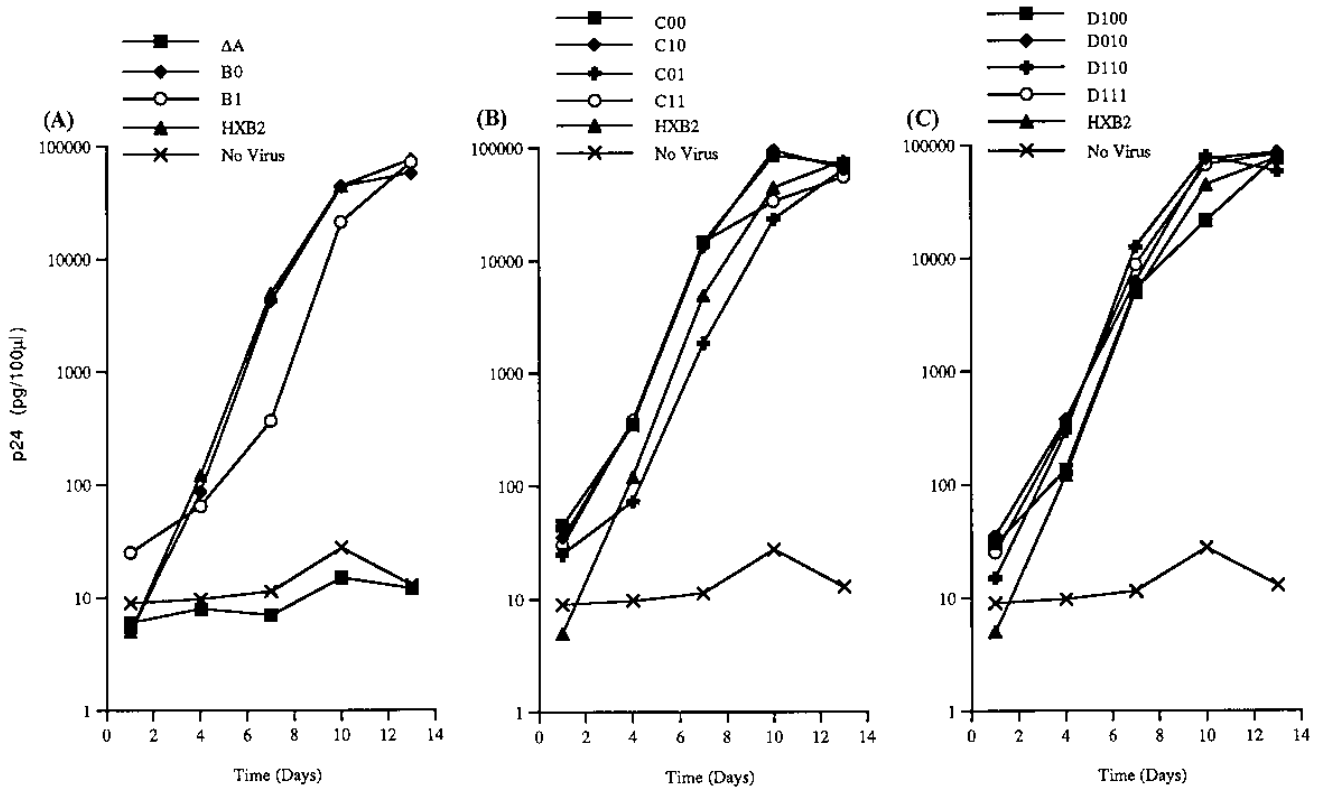

FIG. 1. Effect of V2 mutations on virus growth rate. The 8166 T-cell line was infected with wt and mutant viruses at a multiplicity of 0.01. Infection was monitored by the measurement of soluble p24 antigen as described previously (52). Data from ΔA and B mutants are shown in panel A, C mutants are in panel B, and D mutants are in panel C.

within the N-terminal side of the V2 loop, showed similar affinities for all the expressed proteins. In contrast, MAb 12b, specific for a linear epitope within the V2 apex, showed a 12- and 255-fold reduced binding for the C10 and D100 proteins, respectively (Table 2). Furthermore, all of the MAbs specific for conformation-dependent V2 epitopes failed to saturate the mutant gp120 proteins at concentrations up to 100-fold higher than that required to saturate wt gp120. Comparable results were obtained for recognition of detergent-solubilized viral gp120 from the C and D mutants, indicating that the recombinant protein had properties similar to those of the viral protein (data not shown). Clearly, the changes introduced affect local conformation of the apex and C-terminal region of V2. Both sCD4 and MAbs mapping to epitopes overlapping the CD4 binding site (39.13g, 654, and 589) demonstrated a greater affinity for the C10 and D100 mutant glycoproteins, suggesting that mutations in V2 influence the global conformation of the molecule and increase accessibility of the receptor binding site (Table 2 and Fig. 2). Two MAbs (33/50a/6c and 62/41) recognizing V2-independent epitopes showed increased binding to the mutant proteins (Table 2). In contrast, MAbs specific for V3 (10/54ow and ICR41), C4 (55/45b/3a and 38.1a), and some discontinuous epitopes (55/16/2d) bound equivalently to all of the proteins tested (Table 2).

The increased affinity of the mutant gp120 proteins for sCD4 may be expected to result in an altered growth rate(s) in tissue culture. However, with the exception of ΔA, all of the mutant viruses replicated equivalently (Fig. 1). This may be for a number of reasons. First, HXB2 is a T-cell adapted clone which exhibits fast growth kinetics in cell culture, such that processes independent of CD4 affinity may be limiting. Second,

TABLE 2. Antigenic characterization of V2 mutant soluble glycoproteins

Epitope[a]	Ligand	Concn of MAb giving half-maximal binding to the gps (mg/ml)		
		B1 (wt)	C10	D100
LV2	10/76b	0.0042	0.0034	0.0033
	11/4c	0.05	0.02	0.05
	12b	0.0047	0.046	1.02
CV2	11/68b	0.04	5.2	8.4
	66c	0.16	9.4	14.6
	66a	0.12	3.8	7.7
	55/46/1e	0.12	NS[b]	NS
LV3	10/54 ow	0.047	0.026	0.033
CV3	ICR41	0.31	0.12	0.18
CD4 binding site	sCD4	0.192	0.052	0.041
	39.13g	0.13	0.047	0.047
	589	0.14	0.019	0.019
	654	0.13	0.047	0.047
LC4	55/45b/3a	0.039	0.031	0.021
	38.1a	0.017	0.017	0.013
C	55/16/2d	0.021	0.026	0.027
	33/50a/6c	1.2	0.31	0.33
	2G12	0.05	0.03	0.05
	62/41	NS	0.81	0.75

[a] L, linear epitope; C, conformation-dependent epitope.
[b] NS, no saturation of antigen.

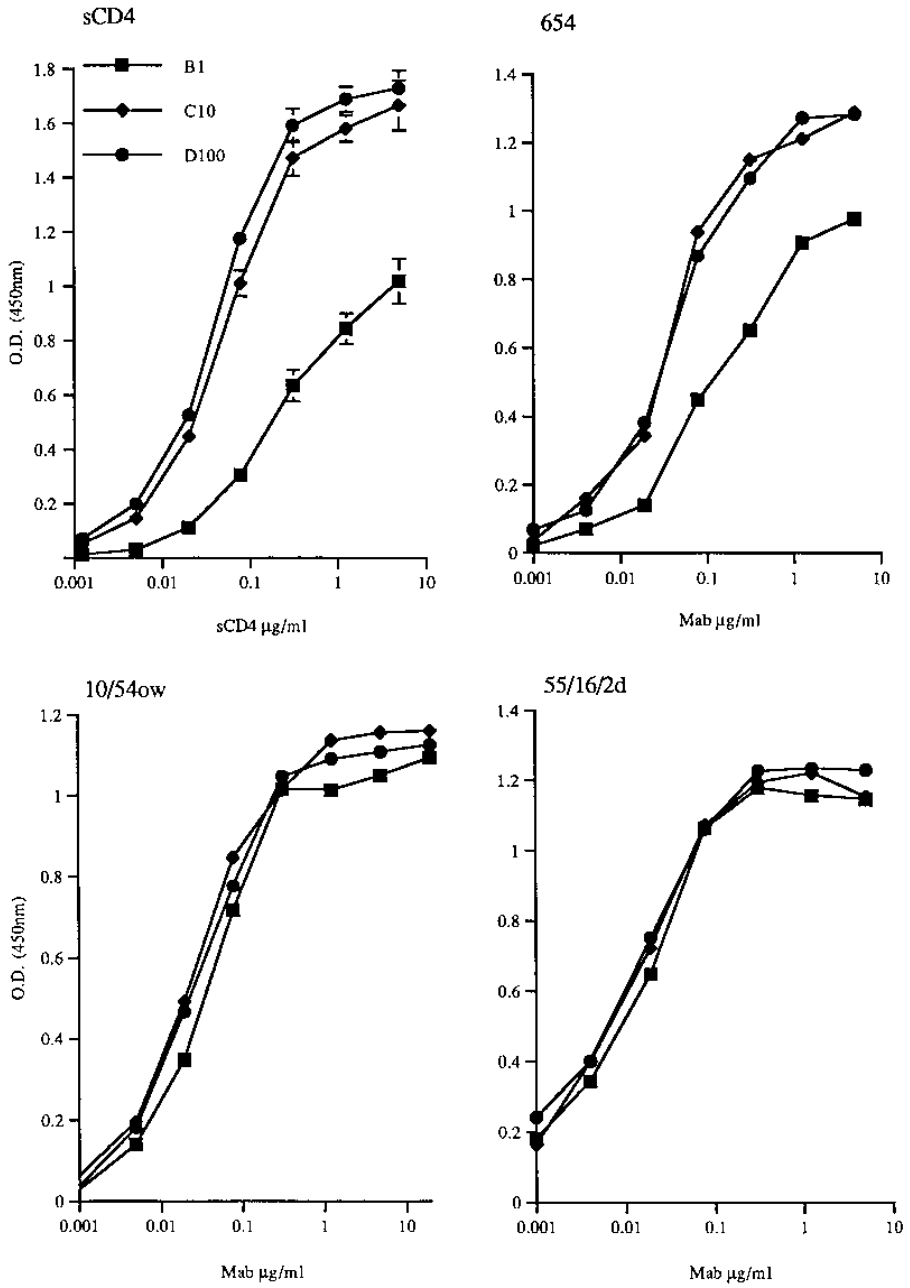

FIG. 2. Antigenic characterization of wt (B1), C10, and D100 gp120 proteins. The gp120 open reading frame was amplified and cloned into pCDNA.3 as described previously (47). Transient transfection of the plasmids into 293 cells yielded soluble gp120 in the range of 1 to 2 mg/ml (47). Soluble B1, C10, and D100 gp120 proteins were assessed in a capture enzyme immunoassay (52) for their ability to bind sCD4; human MAb 654, specific for a discontinuous epitope overlapping the CD4 binding site; rat MAb 10/54 specific for a linear epitope in V3; and rat MAb 55/16/2d, recognizing an undefined conformation-dependent epitope in gp120.

the interaction between sCD4 and soluble gp120 may not accurately reflect the events occurring between oligomeric gp120/gp41 and membrane-associated CD4, such that results obtained using these particular experimental techniques should be interpreted with care.

We (40, 52) and others (63) have reported that the interaction of sCD4 with gp120 results in conformational changes within the V2 loop, resulting in the occlusion of some epitopes. Such a change(s) may be essential for a fusion-competent state; we therefore compared the ability of sCD4 to modulate V2 epitopes in the wt and mutant proteins. Proteins were incubated with a saturating concentration of sCD4 (5 μg/ml), and their ability to bind the V2-specific MAbs 11/4c and 11/68b was measured. The conformation-dependent epitope recognized by 11/68b is known to be sensitive to sCD4 modulation, whereas that of 11/4c is not. We found that sCD4 inhibited the binding of 11/68b to the wt protein but not to either of the mutant proteins (Fig. 3). These data demonstrate that the C10 and D100 mutant V2 loops do not undergo CD4-induced conformational change(s), as measured in this assay, suggesting that interaction(s) between the CD4 binding site and V2 were reduced compared with that of the wt. sCD4 had a minimal

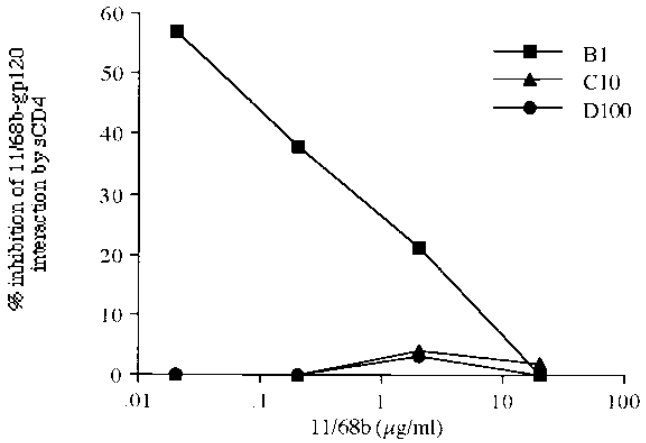

FIG. 3. Soluble CD4-induced conformational change(s) in wt and V2 mutant gp120 proteins. Soluble B1, C10, and D100 gp120 proteins were incubated with a saturating concentration of sCD4 (5 μg/ml), and their subsequent ability to bind MAb 11/68b was measured. The ability of sCD4 to inhibit the MAb-gp120 interaction is shown.

effect on the ability of 11/4c to bind either wt or mutant proteins (data not shown). Since both C10 and D100 viruses replicate as efficiently as the wt, these data suggest that sCD4-induced modulation of V2, as measured by the masking of epitopes recognized by conformation-dependent MAbs, is not an essential step in the fusion process. However, the inability of ΔA virus to enter HeLa CD4 cells suggests that some post-CD4 binding steps are affected by this deletion and are essential for fusion to occur. Moreover, Wyatt and colleagues (63) suggested that modulation of epitopes recognized by the human neutralizing MAbs 17b and 48d by the V2 stem region may be a critical step(s) leading to the fusion of the virus and cell membranes. Thus, the V2 region may be involved in multiple conformational changes induced by CD4 binding, some of which are essential for fusion-related events to occur.

Sensitivity of mutant V2 viruses to neutralization. In order to measure the effect of V2 changes on the overall oligomeric envelope complex, mutant viruses were tested for their sensitivity to neutralization. We monitored the ability of sCD4 and MAbs specific for epitopes within V2 (11/68b, 55/46, 10/76b) (40, 52), V3 (ICR41) (47), the CD4 binding site (39.13g) (47), the C-terminal region of gp120 (2G12) (6), and gp41 (2F5) (13) to neutralize both wt and mutant viruses (Table 3). MAbs 10/76b, 2G12 and 2F5 were able to neutralize wt and mutant viruses equivalently, in agreement with MAb-gp120 binding ELISA data (Table 2). MAbs 11/68b and 55/46, which showed reduced affinity for the mutant proteins compared with wt (Table 2), were unable to neutralize any of the C or D mutants. It is interesting to note that the C and D mutants required up to fourfold more sCD4 to be neutralized. In agreement with this data, all of the C and D mutants were resistant to neutralization by a MAb specific for the CD4 binding site, demonstrating a 16-fold reduction in sensitivity. These data demonstrate that increasing the length of the V2 region affects accessibility of the CD4 binding site both to MAbs and to soluble forms of the receptor. These data are consistent with that of Koito and colleagues (34) who reported that an SF-2 clone chimeric for the SF-162 V2 region was resistant to neutralization by sCD4. One interpretation of these results is that the longer V2 mutant sequences show a reduced interaction(s) with the conserved gp120 core, thereby enhancing accessibility of the CD4 binding site. These data are consistent with a model in which the V2 loop folds into the proximity of the C4 domain (23, 58) and thus partially masks this region from sCD4. The C4 region is known to be a component of the receptor binding site (31). In support of this model, deletion of the V1-V2 loops has been shown to increase exposure of epitopes overlapping the CD4 binding site (28, 63). The decreased sensitivity of mutant viruses to neutralization by sCD4 despite the increased affinity exhibited by the C10 and D100 gp120 proteins for sCD4 is difficult to interpret (Table 3 and Fig. 3). One difference between the two experiments is that sCD4, by definition, is not membrane associated such that other membrane proteins/co-receptors are absent (25). To date, there is no molecular model to explain the resistance of primary virus isolates to sCD4 neutralization despite the ability of some monomeric primary virus glycoproteins to bind sCD4 with affinities comparable to that of gp120 cloned from T-cell-adapted viruses (42, 46, 61). One interpretation of these data is that primary gp oligomeric conformation is different to that of T-cell-adapted viruses, such that studies with monomeric gp120 do not accurately reflect such differences. However, comparable studies with T-cell-adapted monomeric gp120 do yield information correlating with virus neutralization data (29). It is possible that increasing V2 length in the C and D viruses affects gp120 subunit interactions within the oligomer, resulting in an envelope conformation more similar to that of primary viruses.

In addition, both C and D mutant viruses showed reduced sensitivity to neutralization by the V3 MAb ICR41 (Table 3). However, both C10 and D100 gp120 proteins bound V3 MAbs with affinities comparable to that of wt (Fig. 2 and Table 2). Furthermore, the V3 loops on wt, C10, and D100 proteins were equally sensitive to cleavage by thrombin (12) (data not shown). These data suggest that interactions between V2 and V3 may only be apparent in the oligomeric gp and may depend on interactions between gp subunits. All of the C (C00, C10, C01, and C11) and D (D100, D010, D110, and D111) viruses replicated to comparable levels and were generally equally resistant to neutralization by MAbs specific for the V3 and CD4 binding site regions, suggesting that the additional glyco-

TABLE 3. Neutralization of V2 mutant viruses[a]

Virus	Neutralization titer of ligands (μg/ml)							
	sCD4	39.13g	2G12	2F5	11/68b	55/46	10/76b	ICR41
HXB2	0.12	0.80	0.30	1.25	0.80	0.25	2.50	0.40
B0	0.12	0.80	0.30	1.25	0.80	0.25	2.50	0.40
B1	0.12	0.80	0.30	1.25	0.80	0.25	2.50	0.40
C00	0.25	6.25	0.30	1.25	—[b]	—	2.50	0.80
C10	0.25	6.25	0.30	1.25	—	—	2.50	1.60
C01	0.25	6.25	0.60	1.25	—	—	2.50	0.80
C11	0.50	12.50	0.30	1.25	—	—	1.25	0.80
D100	0.50	6.25	0.30	1.25	—	—	1.25	1.60
D010	0.50	6.25	0.30	1.25	—	—	1.25	1.60
D110	0.50	6.25	0.30	1.25	—	—	2.50	3.20
D111	0.50	12.50	0.30	1.25	—	—	1.25	3.20

[a] Neutralization was assessed using the C8166 line as target cells with determination of p24 antigen production as the end point. MAb and sCD4 dilutions (50 μl) were incubated at 37°C (1 h) with an equal volume of virus in duplicate. Following the virus/antibody incubation, 10,000 cells in a final volume of 75 μl RPMI–10% fetal calf serum were added. The 50% infectious dose (ID_{50}) of the virus stock was determined in parallel, and neutralization was evaluated for wells containing 100 ID_{50} of virus. Neutralization was defined as complete inhibition of virus replication as assessed by p24 antigen production. Similar results were obtained using PBMC as target cells.

[b] —, no neutralization at the greatest concentration of ligand used in the assay (25 μg/ml).

sylation sites had no effect or that the sites were not glycosylated. Western blotting of wt and mutant virus envelope gps failed to show any differences in molecular weight, however, such data are difficult to interpret for such extensively glycosylated proteins (data not shown).

Given the reduced sensitivity of the C and D viruses to neutralization by antibodies specific for the V2, V3, and CD4 binding site, we were interested to know if the viruses were also resistant to neutralization by polyclonal human sera. Ten sera from healthy asymptomatic infected individuals were tested for their ability to neutralize wt, C10, and D100. All of the sera were able to neutralize wt virus, in the 1/640 to 1/1,280 range. However, three sera failed to neutralize the C10 and D100 viruses and the remaining seven sera neutralized the mutant viruses with reduced titers, in the range of 1/80 to 1/320. These data demonstrate that changes within V2 modulate the accessibility of the V3 and CD4 binding site regions. Given that V2 length polymorphism occurs frequently in vivo, it is important to consider the consequences of such changes on the overall gp conformation.

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