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Mapping the CD4 binding site for human immunodeficiency virus by alanine-scanning mutagenesis

(acquired immunodeficiency syndrome/envelope glycoprotein gp120/protein engineering/antiviral therapy)

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ABSTRACT **Infection of mononuclear cells by human immunodeficiency virus (HIV) begins with binding of the viral envelope glycoprotein, gp120, to its receptor, CD4. CD4 contains four extracellular immunoglobulin-like domains, the first of which (V1) is sufficient for HIV binding. V1 contains three sequences homologous to the antigen-complementarity-determining regions (CDR1 to -3) of immunoglobulin variable domains. While all three immunoglobulin CDRs are involved in antigen binding, only amino acids within and flanking the CDR2-like region of CD4 have been shown previously to be involved in gp120 binding. To investigate whether other regions in V1 take part in gp120 binding, we substituted alanine for each of 64 amino acids, including all of the hydrophilic residues in this domain. Mutations at four locations outside the CDR2-like sequence (amino acids 29, 59–64, 77–81, and 85) markedly affected gp120 binding, but not the overall structure of V1 as probed with eight conformationally sensitive monoclonal antibodies. Thus, the gp120-binding site of CD4 is not limited to the CDR2-like sequence and consists of several discontinuous segments. Several amino acids were identified that are critical for the conformation of V1; the importance of these residues suggests some differences in the folding of this domain compared to immunoglobulin variable domains. Three amino acid substitutions were found that increase the affinity for gp120 significantly (1.7- to 2-fold individually and 4.2-fold when combined), suggesting that it may be possible to improve the HIV-blocking ability of CD4-based molecules by increasing their gp120 binding affinity.**

CD4 is a cell surface glycoprotein found mainly on T lymphocytes that associates with class II major histocompatibility molecules on antigen-presenting cells, facilitating antigen recognition by the T-cell receptor. CD4 is also the cellular receptor for human immunodeficiency virus (HIV), binding to the viral envelope glycoprotein, gp120 (1, 2). The finding that CD4 is required for HIV infection of human mononuclear cells has led to the development of CD4-based candidates for AIDS therapy. Some of these compounds block the binding of HIV to cell surface CD4 (3–11), while others target toxins to HIV-infected cells (12, 13). An understanding of the interaction of HIV with CD4 may help improve the efficiency of such CD4-based therapeutics.

CD4 is a member of the immunoglobulin gene superfamily, containing four immunoglobulin variable (V)-like domains (V1–V4) in its extracellular region (14, 15). The V1 domain of CD4, containing the first 92 amino acids, is sufficient for binding to gp120 (16, 17). Several approaches have been taken to define the gp120 binding site of CD4, including random saturation mutagenesis coupled with selection of escape mutants (18), insertional mutagenesis (19), and homolog-scanning mutagenesis (17, 20–23). These studies have identified a single amino acid stretch (residues 40–55) as critical for gp120 binding, leading to the notion that the structure of the gp120-binding site may be relatively simple. However, peptides containing residues 81–92 block HIV-induced syncytium formation (8), suggesting that the gp120-binding site may involve more than one region in V1.

To investigate systematically which amino acids in CD4 are critical for HIV binding, we studied the V1 domain by alanine-scanning mutagenesis (24). We selected amino acids with hydrophilic side chains, which are found generally on the surface of the protein, and several hydrophobic amino acids predicted to be either exposed or important for the conformation of V1, and replaced each of them with alanine. Substitution by alanine may identify contact sites for gp120, since protein–protein interfaces are dominated by amino acid side-chain interactions and since alanine substitution eliminates the side chains beyond the β carbon, without changing the main-chain conformation or imposing extreme electrostatic or steric effects. Moreover, alanine is the most abundant amino acid and can be found in both buried and exposed positions and in various secondary structures (ref. 24 and refs. therein). In addition, substitution of single amino acids should yield results that are easier to interpret than the multiple substitutions, insertions, or deletions used in previous studies.

Our results show that the gp120-binding site of CD4 is complex, with residues likely to contact gp120 found in at least four locations outside the 40–55 region. We also identified several residues that are important for the conformation of the V1 domain and three mutations that increase the gp120 binding affinity significantly and thus may aid the design of CD4-based molecules that block HIV more efficiently.

METHODS

Construction and Expression of CD4 Mutants. A molecular fusion of human CD4 and IgG (CD4 immunoadhesin) was used to construct the mutants and to test them as secreted molecules (9, 11). Mutations were introduced using the polymerase chain reaction (25). The mutants were transiently expressed and secreted by transfection of human embryonic kidney cells (9).

Analysis of gp120 and Monoclonal Antibody (mAb) Binding. Serum-free supernatants from transfected cells were tested for binding of ^{125}I-labeled recombinant gp120 (IIIb isolate of HIV) (3). Dissociation constants (K_d) were determined by Scatchard analysis (26). The binding of murine mAbs to the CD4 immunoadhesins was tested in an ELISA format. MT 151 was from

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Abbreviations: HIV, human immunodeficiency virus; mAb, monoclonal antibody; VL, light-chain variable; CDR, complementarity-determining region.

Boehringer Mannheim, OKT4A (T4A) from Ortho Pharmaceuticals, and anti-Leu-3a (L3a) from Becton Dickinson. mAb 671 was generated against soluble CD4, and mAbs 730, 725, 750, and 752 against wild-type CD4 immunoadhesin.

Modeling the Structure of the V1 Domain of CD4. We modeled the structure of the V1 domain by using the average coordinates of structurally conserved regions (primarily β-sheet) from crystal structures of seven immunoglobulin light-chain V (VL) domains, after superpositioning. The VL domains were [Protein Data Bank code (27) in parentheses] human λ KOL (1FB4) (28) and NEWM (3FAB) (29); human Bence-Jones proteins REI (1REI) (30) and RHE (2RHE) (31); and mouse κ HyHEL-5 (2HFL) (32), MCPC603 (2MCP) (33), and J539 (1FBJ) (34). Conformations of the complementarity-determining region 1 (CDR1)- and CDR2-like regions were taken from VL structures, and that of CDR3, which is larger in CD4 than in immunoglobulins, from loops of proteins unrelated to VL domains. Side-chain conformations were adapted from VL domains or predicted from allowed conformations (35). Several models were subjected to energy minimization using the DISCOVER program (Biosym Technologies, San Diego).

RESULTS AND DISCUSSION

We used a CD4 immunoadhesin construct to test the mutants as secreted molecules. In this molecule, the V1 and V2 domains of CD4 are linked at their carboxyl terminus to the hinge and Fc portion (CH2 and CH3 heavy-chain constant domains) of human IgG1; the resulting polypeptide folds as a homodimeric protein (9, 11). Wild-type and mutant CD4 immunoadhesins were transiently expressed in human embryonic kidney cells and harvested in serum-free cell supernatants. All 64 variants constructed were expressed and secreted, as determined by both radioimmunoprecipitation and immunosorbent assays that recognize the invariant Fc domain (data not shown).

We tested the binding of each mutant to ^{125}I-labeled gp120 (Table 1). The wild-type immunoadhesin exhibited a K_d of 3.6 nM, similar to intact, cell-surface CD4 (22, 36). Mutations in 38 of the positions tested displayed K_d values comparable to that of the wild type (Table 1; Fig. 1), suggesting that those residues are not involved directly in gp120 binding. Alanine substitutions at the other 26 positions tested markedly affected gp120 binding; these residues may interact with gp120 directly but could also affect the binding indirectly, by changing the conformation of V1.

To distinguish between these possibilities, we probed the overall structure of the V1 domain in each mutant with a panel of eight conformationally dependent anti-V1 mAbs. Each of these antibodies binds to native CD4 but not to CD4 that is reduced and alkylated (data not shown). Analysis of mAb binding of each mutant revealed that 12 of the 26

Table 1. Effect of mutations in CD4 on binding to gp120 and to anti-CD4 mAbs

	gp120	mAb binding									gp120	mAb binding							
Mutant*	K_d, nM	151	671	730	T4A	L3a	725	750	752	Mutant*	K_d, nM	151	671	730	T4A	L3a	725	750	752
wt	3.6	+++	+++	+++	+++	+++	+++	+++	+++	K50A	3.9	+++	+++	+++	+++	+++	+++	+++	+++
K1A	4.0	−	+++	+++	+++	+++	+++	+++	+++	L51A	28.9	++	+	−	+	++	−	−	+
K2A	3.5	+++	+++	+++	+++	+++	+++	+++	+++	N52A	11.9	+++	+++	+++	+++	+++	+++	+++	+++
K7A	19.4	++	−	−	+	++	−	−	−	D53A	6.0	+++	+++	+++	+++	+++	+++	+++	+++
K8A	4.3	++	+++	++	++	+++	+++	+++	+++	R54A	nbd	−	−	−	−	−	−	−	−
D10A	3.6	+++	+++	+++	++	+++	+++	+++	+++	D56A	3.2	+++	+++	+++	+++	+++	+++	+++	+++
T11A	3.6	+++	+++	+++	+++	+++	+++	+++	+++	S57A	nbd	+	−	−	−	−	−	−	+
E13A	nbd	+	−	−	−	+	−	−	+	R58A	4.5	+++	+++	+++	−	+++	+++	+++	+++
T15A	6.2	+++	+++	+++	+++	+++	+++	+++	+++	R59A	26.2	+++	+++	+++	+++	++	+++	+++	+++
T17A	2.9	+++	+++	+++	+++	+++	+++	+++	+++	S60A	3.1	+++	+++	+++	+++	+++	+++	+++	+++
S19A	3.6	+++	+++	+++	+++	+++	+++	+++	+++	W62A	nbd	++	−	−	+	+	−	−	−
Q20A	3.5	+++	+++	+++	+++	+++	+++	+++	+++	D63A	2.1	+++	+++	+++	+++	+++	+++	+++	+++
K21A	2.9	+++	+++	+++	++	+++	+++	+++	+++	Q64A	7.6	+++	+++	+++	+++	+++	++	++	+++
K22A	5.4	+++	+++	+	+++	+++	+++	++	+++	N66A	3.4	+++	+++	+++	+++	+++	+++	+++	+++
S23A	5.9	+++	+++	+++	+++	+++	+++	+++	+++	K72A	3.5	+	+++	++	+	++	++	+++	+++
Q25A	3.8	+++	+++	+++	+++	+++	+++	+++	+++	N73A	3.0	++	+++	++	++	++	++	+++	+++
H27A	5.8	+++	+++	+++	+++	+++	+++	+++	+++	K75A	4.7	+++	+++	+++	+++	+++	+	+++	+++
W28A	nbd	+	−	−	−	+	−	−	+	E77A	9.3	++	+++	++	+	++	+++	+++	+++
K29A	9.8	+++	+++	+++	+++	+++	+++	+++	+++	D78A	nbd	+	−	+	−	+	−	−	+
N30A	4.8	+++	+++	+++	+++	+++	+++	+++	+++	S79A	nbd	+++	−	−	++	++	−	−	+
S31A	4.3	+++	+++	+++	++	+++	+++	+++	+++	D80A	nbd	++	−	++	+	+	−	−	++
N32A	4.9	+++	+++	+++	+++	+++	+++	+++	+++	T81A	nbd	++	+++	+++	+	+++	+++	+++	+++
Q33A	4.3	+++	+++	+++	++	+++	+++	+++	+++	Y82A	31.8	+	−	−	−	−	−	−	−
K35A	6.2	+++	+++	+++	+++	−	+++	+++	+++	E85A	33.6	+++	+++	+++	+++	++	+++	+++	+++
I36A	nbd	+	++	−	−	+	−	+++	++	V86A	3.2	+++	+++	+++	+++	+++	+++	+++	+++
L37A	nbd	++	++	+	−	+	−	++	++	E87A	5.2	+++	+++	+++	+++	+++	+++	+++	+++
N39A	7.9	+++	+++	+++	+++	++	+++	+++	+++	D88A	3.2	+++	+++	+++	+++	+++	+++	+++	+++
Q40A	1.8	+++	+++	+++	+++	+++	+++	+++	+++	Q89A	4.8	+++	+++	+++	+++	+++	+++	+++	+++
S42A	3.6	+++	+++	+++	+++	+++	+++	+++	+++	K90A	3.9	+++	+++	+++	+++	+++	+++	+++	+++
L44A	21.4	+++	+++	+++	+	+	++	++	+++	E91A	2.9	+++	+++	+++	+++	+++	+++	+++	+++
T45A	2.8	+++	+++	+++	+++	+++	+++	+++	+++	E92A	3.7	++	+++	++	+	++	++	+++	+++
K46A	40.3	++	++	+	+	+	+	+	++	Q94A	3.0	−	++	+	++	+++	++	++	++
S49A	10.1	+++	+++	+++	++	+++	+++	+++	+++										

All the hydrophilic and several hydrophobic amino acids in the V1 domain of CD4 were replaced individually with alanine by site-directed mutagenesis and expressed as CD4 immunoadhesins by transient transfection in human embryonic kidney cells. The dissociation constants (K_d) for gp120 binding were determined as described under *Methods*. Standard errors for K_d were not greater than 10% of the means reported (n = 3). nbd, No binding detected. The relative binding of each of eight mAbs to CD4 mutants as compared to binding to the wild type is also depicted: −, <10%; +, 10–20%; ++, 21–50%; +++, 51% or more of the binding to wild type.

*Names indicate residue (one-letter symbol and position number) replaced by alanine (A). wt, Wild type.

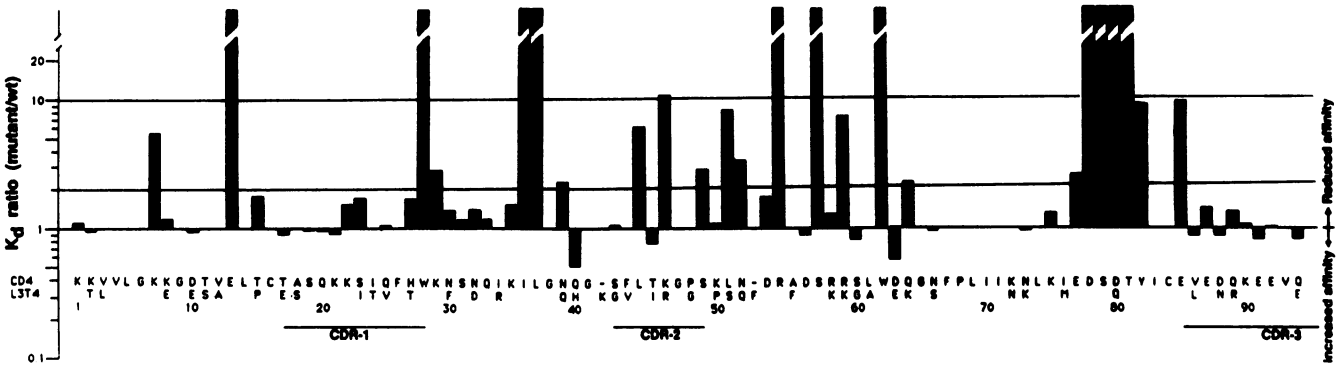

FIG. 1. Effect of alanine substitution of individual amino acids in the V1 domain of CD4 on gp120 binding. Histogram depicts the ratio of the K_d of each mutant to the K_d of wild-type (wt) CD4 (see Table 1 for actual K_d values). Values greater or smaller than 1 indicate a decrease or an increase, respectively, in the affinity for gp120. Amino acid sequences of the V1 domain of human (CD4) and murine (L3T4) CD4 were aligned according to Maddon *et al.* (15). Regions homologous to CDRs of immunoglobulin light chains are also indicated.

mutants that displayed altered in gp120 binding (positions 29, 39, 40, 44, 49, 52, 59, 63, 64, 77, 81, and 85) bound most of the mAbs as well as the wild type, indicating that the overall structure of V1 was retained (Table 1). These residues, therefore, appear to be involved directly in binding to gp120. Ten other mutants (positions 7, 13, 28, 54, 57, 62, 78, 79, 80, and 82) exhibited dramatic reductions in binding to most or all mAbs (Table 1), suggesting that mutations in these positions alter the conformation of V1 and therefore may affect gp120 binding indirectly. Four mutants (positions 36, 37, 46, and 51) exhibited intermediate reductions in mAb binding (Table 1); the reduction in gp120 binding in these mutants also may be due to changes in conformation.

We and others (18, 20, 21, 37) have constructed models for the structure of the CD4 V1 domain, based on the known structures of immunoglobulin VL domains. Within the limitations of such models, one can draw certain inferences from the basic similarity of CD4 to VL domains. Much of the sequence of CD4 is predicted to fold in two β-pleated sheets, which lie parallel to each other in an immunoglobulin-like "sandwich" (Fig. 2*A*). The previously known mutations that affect gp120 binding are located in an exposed loop homologous to CDR2 of a VL domain (18–22, 38). This loop contains two antiparallel β-strands (residues 36–45 and 48–53). To reconcile our data with a VL-domain-like model of CD4, one could postulate that the CDR2-like loop assumes a conformation different from that predicted by a simple translation of VL structure. Because Glu-77 and Thr-81 appear to form direct contacts with gp120 (see above), it is possible that the segment of amino acids 77–81, in which these residues are found, is exposed more than the homologous segment in VL domains. Indeed, the 77–81 region is accessible significantly more if the small loop containing Ile-36 and Leu-37 is folded over toward residues Ile-83, Phe-98, and Leu-100 (these five residues are hydrophobic in CD4 from various species), rather than outward, as is the case in immunoglobulins (Fig. 2*B*). This would result in a different conformation of the CDR2-like loop, as Ile-36 and Leu-37 are at the base of this loop. That Ile-36 and Leu-37 affect the conformation of the CDR2-like loop is consistent with the observation that substitution of these residues abolishes gp120 binding completely: because many amino acids in the CDR2-like region appear to interact with gp120 directly, a change in the conformation of this loop may obstruct the CD4–gp120 interaction.

Within the CDR2-like region, mutations in Ser-39, Gln-40, Leu-44, Lys-46, Ser-49, Leu-51, and Asn-52 resulted in marked reductions in gp120 binding (Table 1; Fig. 1). These residues are predicted to be exposed (Fig. 2*C*) and therefore are likely to interact directly with gp120. However, since mutants K46A and L51A also exhibited significant reductions in mAb binding (Table 1), it is possible that Lys-46 and Leu-51 are important for local conformation as well. The residues outside the CDR2-like loop that appear to contact gp120 were found in four regions. The first includes Arg-59, Asp-63, and Gln-64, which are predicted to be external and adjacent to the CDR2-like loop (Fig. 2*C*), consistent with the conclusion that they may interact with gp120. The neighboring residues Ser-57 and Trp-62 are predicted to be buried (Fig. 2*B*) and their substitution probably affects gp120 binding through changes in conformation, as indicated by mAb binding. In the second region, residues 77–81, Glu-77, and Thr-81 appear to interact with gp120 directly, while Asp-78, Ser-79, and Asp-80 appear to be important for conformation. These findings support two predictions of our model: Glu-77 and Thr-81 are the most accessible residues in the 77–81 segment, and Asp-78 forms an important salt bridge with Arg-54, an interaction found for the homologous residues in most VL domains (R54A, like D78A, displays a total loss of gp120 binding).

Mutations in Lys-29 and Glu-85 also appear to affect gp120 binding directly (Table 1). Consistent with this conclusion, Lys-29 is predicted to be external and in a β-strand immediately adjacent to the CDR2-like loop, while Glu-85 is located externally as well, in the β-strand adjacent to Lys-29. These residues also might form a salt bridge together (Fig. 2*C*).

Mutations in Lys-7, Glu-13, Trp-28, and Tyr-82 reduced the binding to all eight mAbs, indicating that these residues are important for the conformation of V1 (Table 1). Consistent with this notion, the model predicts that Lys-7 and Glu-13 are capable of forming a salt bridge and that Trp-28 and Tyr-82 lie internally, Trp-28 abutted against the conserved disulfide bond and Tyr-82 hydrogen-bonded to the V1 backbone (Fig. 2*B*). These residues are conserved between CD4 and VL domains, further suggesting that they may be important structurally.

Two of the alanine substitution mutants, Q40A and D63A, exhibited significantly higher affinity for gp120 than the wild-type CD4 (2.0- and 1.7-fold, respectively; Table 2). Similarly, a leucine substitution mutant, Q89L, constructed as part of a more extended mutational analysis (A.A., S.A.M., and D.J.C., unpublished results), bound gp120 with 1.7-fold higher affinity than the wild-type CD4. The double mutant Q40A/D63A exhibited a 2.3-fold increase in affinity and the triple mutant Q40A/D63A/Q89L exhibited a 4.2-fold increase in affinity (Table 2). As Gln-40 and Asp-63 are located externally in segments that appear to interact with gp120, their side chains may hinder the normal interaction of gp120 with CD4. In contrast, Gln-89 does not appear to be involved directly in binding, as its replacement by alanine has no effect (Table 1). However, a leucine at this position may form a direct contact with gp120 and thereby stabilize bind-

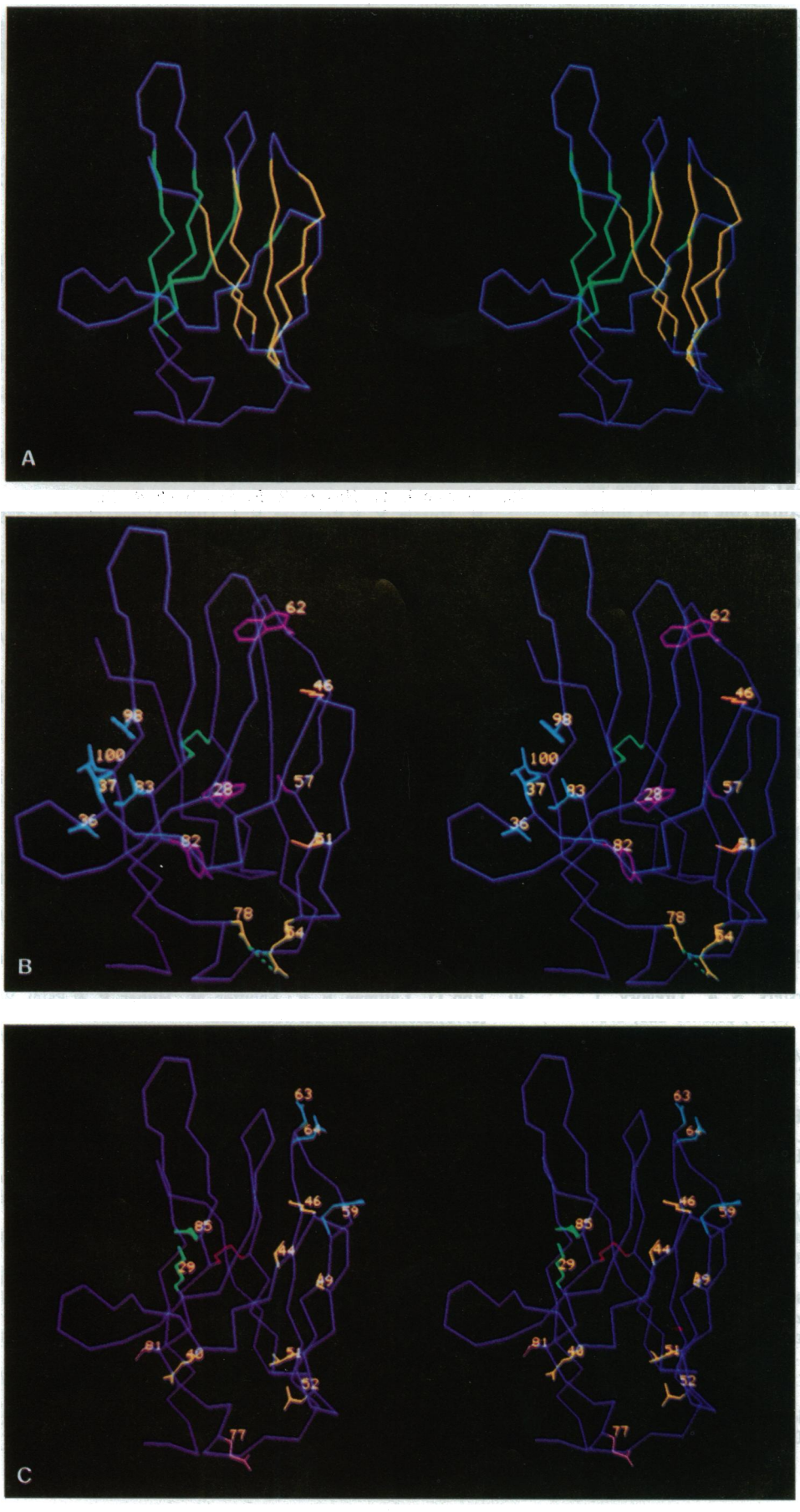

FIG. 2. Proposed model for the structure of the V1 domain of CD4. (*A*) Stereo pair depicting the β-carbon backbone, to illustrate the overall structure of the V1 domain. The core of this domain is composed of two β-sheets. One sheet, shown in yellow, is formed primarily by residues in four β-strands, termed A, B, E, and D (residues 4–8, 13–18, 65–71, and 55–61, respectively) according to the immunoglobulin nomenclature. Another sheet, shown in green, is formed primarily by residues in three β-strands, termed C, F, and G (residues 25–29, 82–87, and 96–102, respectively). These β-sheets are connected by a large loop, formed by strands C′ (residues 40–46) and C″ (residues 47–53). Residues in strands C′ and C″ that may take part in the two β-sheets are shown in green and yellow (residues 45 and 49, respectively). (*B*) Stereo pair showing the same backbone at a slightly rotated angle and highlighting the predicted side-chain conformations of residues that appear to be important for the conformation of the V1 domain. (*C*) Stereo pair highlighting the predicted side-chain conformations of residues that appear to contact gp120 directly (see text).

ing. While it is unlikely that gp120 residues involved in binding to CD4 diverge in various strains of HIV, it remains to be tested whether these mutations will affect similarly the binding to other gp120 isolates.

Table 2. Mutations that increase CD4–gp120 binding affinity

Mutant	K_d, nM	Fold increase
Wild type	3.60 ± 0.20	—
Q40A	1.80 ± 0.10	2.0
D63A	2.10 ± 0.10	1.7
Q89L	2.13 ± 0.05	1.7
Q40A/D63A	1.55 ± 0.07	2.3
Q40A/D63A/Q89L	0.86 ± 0.04	4.2

The K_d values (means ± SEM, $n = 3$) were determined as in Table 1. Multiple point mutants were generated by using single or double point mutants as templates while introducing an additional mutation through polymerase chain reaction primers.

In conclusion, our results show that the gp120-binding site of CD4 consists of several discontinuous segments, which can be modeled to fold in space into a relatively compact region of CD4. In addition, we have identified several residues that appear to be important structural determinants for the conformation of the V1 domain. These data suggest that the CDR2-like loop is folded differently in CD4 than in immunoglobulins and that the 77–81 segment is more exposed and plays an important structural role in CD4, unlike the homologous region in VL domains. Finally, the observation that certain CD4 mutants have a markedly increased gp120 binding affinity and that such mutations can be combined to produce molecules that bind gp120 yet more strongly may be useful in the development of CD4-based molecules that are more efficient in blocking HIV infection or in targeting the destruction of HIV-infected cells.

Note Added in Proof. During preparation of this manuscript, Brodsky *et al.* (39) reported a mutational analysis of the gp120 binding site of CD4. Their findings are consistent with our conclusion that the binding site is discontinuous and not limited to amino acids in the CDR2-like region.

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