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Immunological Evidence for Interactions between the First, Second, and Fifth Conserved Domains of the gp120 Surface Glycoprotein of Human Immunodeficiency Virus Type 1

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We have used a combination of genetic and immunological techniques to explore how amino acid substitutions in the second conserved (C2) domain of gp120 from human immunodeficiency virus type 1 (HIV-1) affect the conformation of the protein. It was reported previously (R. L. Willey, E. K. Ross, A. J. Buckler-White, T. S. Theodore, and M. A. Martin. J. Virol. 63:3595–3600, 1989) that an asparagine-glutamine (N/Q) substitution at C2 residue 267 of HIV-1 NL4/3 reduced virus infectivity, but that infectivity was restored by a compensatory amino acid change (serine-glutamine; S/N) at residue 128 in the C1 domain. Here we show that the 267 N/Q substitution causes the abnormal exposure of a segment of C1 spanning residues 80 to 120, which compromises the integrity of the CD4-binding site. The reversion substitution at residue 128 restores the normal conformation of the C1 domain and recreates a high-affinity CD4-binding site. The gp120 structural perturbation caused by changes in C2 extends also to the C5 domain, and we show by immunological analysis that there is a close association between areas of the C1 and C5 domains. This association might be important for forming a complex binding site for gp41 (E. Helseth, U. Olshevsky, C. Furman, and J. Sodroski. J. Virol. 65:2119–2123, 1991). Segments of the C1 and C2 domains are predicted to form amphipathic alpha helices. We suggest that these helices might be packed together in the core of the folded gp120 molecule, that the 267 N/Q substitution disrupts this interdomain association, and that the 128 S/N reversion substitution restores it.

The surface glycoprotein gp120 of human immunodeficiency virus type 1 (HIV-1) mediates attachment to the cell surface glycoprotein CD4 in the initial stage of infection of $CD4^+$ cells by this pathogenic lentivirus (19, 20, 23). The mature gp120 glycoprotein comprises approximately 480 amino acids, about 30 of which are asparagine residues with N-linked carbohydrate chains that, in toto, account for nearly 50% of the total molecular weight of the protein (10, 17). There is also evidence for O-linked glycosylation on several, undefined serine residues (4, 14). Analyses of gp120 sequences from different strains of HIV-1 allowed the division of the protein into conserved (C) and variable (V) domains (17, 21, 29, 34). Biochemical techniques have defined the nine pairs of disulfide bonds that stabilize the gp120 structure (17). The disulfide bond pattern of gp120 shows that the first four variable segments (V1 to V4) form simple or complex loops (17). Mapping of linear antibody epitopes indicates that the variable loops are mostly exposed on the monomeric form of the HIV-1 gp120 glycoprotein. However, of the conserved gp120 regions, only limited segments of C1, C4, and C5 are exposed on the native gp120 monomer, and these conserved regions are poorly accessible on the assembled gp120-gp41 tetramer (25). Secondary structural information on gp120, based on infrared spectroscopic analysis or computer modeling techniques, is also available (7, 13).

Our understanding of the tertiary structure of gp120 is, however, limited; the flexibility and extensive glycosylation of gp120 have thus far precluded its crystallization and the subsequent examination of its structure by X-ray spectroscopy. We are therefore using mutational analysis coupled with immunochemical procedures to obtain additional information on the interactions between the different domains of gp120 (25–27, 35–38, 42, 45, 46). For example, we have demonstrated that there is a close association between the V3 and C4 domains of HIV-1 gp120 (27, 46) which is also found in gp120 from simian immunodeficiency virus (28). In addition, we have shown that a reversion mutation in C1 compensated for a replication-inactivating mutation in C2, implying an interaction between these regions of the protein (42, 43). Furthermore, functional links between the V2 and C4 domains of gp120 (12, 26) and between the C2 and V3 domains (5, 41, 42) have been reported. Here we explore the association between the C1 and C2 domains of gp120, and we provide evidence to suggest that there is also an interaction between the C1 and C5 domains.

MATERIALS AND METHODS

Sources of gp120 and monoclonal antibodies (MAbs). Recombinant gp120 expressed in Chinese hamster ovary cells (batch 42) from the BH10 clone of HIV-1 LAI by Celltech Ltd., Slough, England (24), was obtained as purified protein

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from the reagent repository of the UK Medical Research Council AIDS Directed Programme (ADP reagent repository). The production of mutants of HxBc2 gp120, their expression as proteins in the form of culture supernatants from transfected COS cells, and their use in mapping antibody epitopes have been described previously (15, 16, 25–27, 31, 35–38). The preparation of infectious molecular clones containing gp120 amino substitutions on the background of the NL4/3 clone has been described elsewhere (2, 41–44). Briefly, HeLa cells were transfected with the wild-type or different mutant proviral plasmid DNAs from the NL4/3-based clones, and cell-free culture supernatants containing both virion-associated and soluble gp120 were treated with 1% Nonidet P-40 (NP-40) nonionic detergent to provide a source of gp120. The amino acid numbering system used throughout is based on the HxBc2 gp120 sequence, with the first amino acid of the mature protein being designated residue 31. However, there are minor sequence variations between the HxBc2 and NL4/3 clones of HIV-1 LAI (29); for clarity, we have retained the designations of several NL4/3 mutants used in the original reports (41–44). Note that NL4/3 gp120 residues 128, 267, and 308 correspond in position to HxBc2 residues 128, 262, and 304.

Details of all MAbs used are provided elsewhere (25). Continuous epitopes for these MAbs are as defined previously (25) or in some cases as refined by more-detailed analysis using shorter peptides (18). Donors of or references for the murine antibodies used for Fig. 1 to 3 and Tables 1 and 2 were as follows: M85, M90, M91 (rat MAb), M92, and M96 (rat MAb), Fulvia di Marzo Veronese (8); 133/192 and 135/9, Matthias Niedrig (30); T2.1, T7.1, and T9 (3), Lennart Åkerblom, Britta Wahren, and Jorma Hinkula; B2 (1, 18); 660-178, Gerard Robey and Mary Pinkus (Abbott Laboratories); 110.C, François Traincard (Hybridolabs, Institut Pasteur); 1C1, Repligen Inc.; 4D4#85 and 6D5, Steve Nigida and Larry Arthur. The following MAbs were obtained from the ADP reagent repository via the manager, Harvey Holmes. Their original donors were as follows: CRA-1 (ADP 323), Mark Page; 4A7C6 (ADP360) Richard Tedder; 187.2.1 (ADP 332), Claudine Bruck and Clothilde Thiriart (39). Human MAbs (HuMAbs) C11 and 212A have been briefly described elsewhere (32). Soluble CD4 (sCD4) was obtained from Biogen Inc., Cambridge, Mass. (11).

Reactivities of MAbs with monomeric gp120. Reactivity was determined essentially as described elsewhere (22, 24–27). Briefly, BH10 gp120 (25 ng/ml) or gp120 from culture supernatants diluted 1:3 to 1:15 was captured onto a solid-phase via its carboxy terminus, using sheep polyclonal antibody D7324 (Aalto BioReagents, Dublin, Eire). The gp120 was added either in Tris-buffered saline (TBS) containing 10% fetal calf serum or, when culture supernatants were used, in the same buffer plus 1% NP-40 nonionic detergent. MAbs were added to gp120 in TBS containing 2% nonfat milk powder and 20% sheep serum (TMSS buffer), further supplemented with 0.5% Tween 20 nonionic detergent for experiments on NP-40-treated gp120 (TMTSS buffer). sCD4 binding to gp120 was detected with murine anti-CD4 MAbs (22).

Effects of gp120 amino acid substitutions on MAb binding to monomeric gp120. The procedure was performed with an enzyme-linked immunosorbent assay (ELISA) format essentially as described previously (25–27). Briefly, culture supernatants (100 μl) from COS-1 cells transfected 48 h earlier with 10 μg of plasmid pSVIIIenv expressing either wild-type or mutant HIV-1 (HxBc2) envelope glycoproteins were supplemented with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.0). The gp120 molecules in the supernatants were captured onto a solid phase via antibody D7324 in the absence of detergent, then a fixed concentration of MAb was added in TMSS buffer, and the amount of bound MAb was determined as described above. The MAb concentration was selected from prior titration curves to give approximately 75% of the level of binding at saturating MAb concentrations, or the highest possible binding commensurate with a low background if saturating binding could not be achieved. Absorbance (optical density at 492 nm [OD_{492}]) values were generally in the range 0.3 to 0.8 for each mutant with most MAbs. Because this level of binding is nonsaturating for our detection systems, we were able to seek enhancing as well as inhibitory amino acid substitutions. All the gp120 mutants were accommodated in a single ELISA plate.

To assess the significance of the binding data, we measured, in quadruplicate plates, the reactivities of the gp120 mutants with a pool of HIV-1^+ human sera, binding of the serum pool being carried out in the presence of 0.5% Tween 20 to maximize reactivity with as many epitopes as possible. After correction for background (mock transfection supernatant), the mean OD_{492} value for each mutant was calculated as a reference value. Each test MAb was assessed for binding to the mutant panel in triplicate, and the mean OD_{492} value was determined for each mutant. The OD_{492} ratio of test MAb to reference serum was then determined for each mutant, and the average value of this ratio for the whole panel was then calculated (mean ± standard deviation [SD]), omitting values close to zero (amino acid changes that abolish binding) or very much greater than the mean value to avoid skewing of the data. This value defines the mean binding ratio for each MAb across the mutant panel (see Table 1). The mean binding ratio is then redefined as equal to 1.00, and the binding ratio for each individual gp120 mutant is expressed relative to the mean binding ratio. Ratios of <0.5 or >1.5 are considered to be indicative of inhibitory or enhancing mutants, respectively.

sCD4 binding to virions. The assay was performed essentially as described previously (44). Briefly, HeLa cells were transfected with proviral plasmid DNA from wild-type NL4/3 or mutants thereof and labeled with [^{35}S]methionine for 24 h. Radiolabeled virions were purified by pelleting in an ultracentrifuge, resuspended in RPMI 1640 medium, and incubated with CD4 immunoglobulin G (IgG) for 0.5, 1, and 3 h at 37°C. The amount of gp120 bound to CD4 IgG at the different times was determined (44).

RESULTS

Amino acid substitutions in the C2 domain of gp120 increase the exposure of antibody epitopes in the C1 domain. We have shown previously that much of the C1 domain of monomeric, nondenatured BH10 or HxBc2 gp120 is fairly accessible for the binding of MAbs to defined, continuous epitopes (25). Furthermore, the binding sites for many of these MAbs are destroyed by amino acid substitutions within the C1 domain, often located within the antibody epitope as defined by mapping with short peptides (18, 25). To explore how amino acid substitutions elsewhere in gp120 influenced the structure of continuous antibody epitopes within the C1 domain, we determined MAb reactivity with a panel of HxBc2 gp120 mutants. MAbs reactive with several continuous epitopes within the amino-terminal region of the C1 domain, spanning residues 31 to 70, tended to be relatively insensitive to distant-site substitutions, and no consistent pattern was observed (data not shown). However, the binding of MAbs to several continuous epitopes spanning amino acids 80 to 120 was strongly enhanced by certain amino acid substitutions in the C2 domain and also

TABLE 1. Amino acid substitutions influencing the binding of MAbs to continuous epitopes in the C1 domain of gp120[a]

Mutant (domain)	Normalized binding ratio				
	4A7C6	T9	133/192	135/9	M90
Down					
76 P/Y (C1)	0.65	1.01	**0.35**	0.82	0.90
88 N/P (C1)	**0.00**	1.74	1.96	2.45	0.57
106 E/A (C1)	0.65	0.61	0.52	**0.47**	0.80
113 D/A (C1)	0.93	0.71	**0.43**	**0.22**	0.88
113 D/R (C1)	0.65	0.80	**0.35**	**0.08**	1.03
117 K/W (C1)	2.05	0.90	**0.35**	**0.43**	0.75
380 G/F (C3)	**0.38**	0.74	0.52	0.75	0.77
420 I/R (C4)	**0.38**	0.68	**0.26**	0.58	0.88
427 W/S (C4)	0.60	1.00	**0.48**	0.80	0.94
Up					
45 W/S (C1)	**1.53**	**1.97**	**1.65**	**1.67**	0.59
88 N/P (C1)	0.00	**1.74**	**1.96**	**2.45**	0.57
102 E/L (C1)	**1.73**	0.99	0.78	0.75	1.04
117 K/W (C1)	**2.05**	0.90	0.35	0.43	0.75
125 L/G (C1)	**1.98**	1.41	**1.52**	1.05	1.30
256 S/Y (C2)	**4.68**	**3.48**	**6.35**	**4.07**	**1.58**
257 T/R (C2)	1.22	1.26	**1.96**	1.37	1.19
257 T/A (C2)	**1.70**	1.32	**1.96**	**1.80**	0.97
257 T/G (C2)	1.40	1.29	1.30	**1.65**	0.84
262 N/T (C2)	**5.65**	**3.52**	**7.30**	**4.65**	1.28
266 A/E (C2)	**1.65**	1.20	**1.91**	1.47	1.18
267 E/L (C2)	0.75	1.16	0.91	0.87	0.94
269 E/L (C2)	**1.50**	1.28	1.22	0.90	0.97
421 K/L (C4)	**1.88**	0.88	1.22	1.10	1.04
475 M/S (C5)	1.48	**1.74**	**1.61**	**1.50**	1.09
485 K/V (C5)	**1.80**	**1.83**	1.22	**1.70**	1.33
491 I/F (C5)	**1.63**	**1.65**	1.39	1.20	1.17

[a] The peptide epitope residues to which the MAbs bound and the mean binding ratios ± SD (normalized to a value of 1; see Materials and Methods) are as follows: 4A7C6, 84 to 89 and 0.40 ± 0.16; T9, 94 to 100 and 0.69 ± 0.20; 133/192, 92 to 96 and 0.23 ± 0.09, 135/9, 108 to 113 and 0.60 ± 0.19; M90, discontinuous and 0.69 ± 0.13. MAbs were tested at the following concentrations: 4A7C6, 1:300 dilution of ascites fluid; T9, 5 μg/ml; 133/192, 2 μg/ml; 135/9, 1 μg/ml; M90, 1:500 dilution of ascites fluid. All gp120 mutants that registered as either down or up mutants for one or more MAbs are listed, with values outside the range of 0.5 to 1.5 being highlighted in boldface to indicate their significance. Note that some mutants are up for one MAb but down for another. Mutants registering as neutral (i.e., within the normalized binding ratio range of 0.5 to 1.5) for all MAbs are not listed but were tested. The complete mutant panel comprised wild type, 36 V/L, 40 Y/D, 45 W/S, 69 W/L, 76 P/Y, 80 N/R, 88 N/P, 102 E/L, 103 Q/F, 106 E/A, 113 D/A, 113 D/R, 117 K/W, 120/121 VK/LE, 125 L/G, ΔV1/V2, 152/153 GE/SM, 168 K/L, 176/177 FY/AT, 179/180 LD/DL, 192–194 YSL/GSS, 207 K/W, 252 R/W, 256 S/Y, 257 T/A, 257 T/R, 257 T/G, 262 N/T, 266 A/E, 267 E/L, 269 E/L, 281 A/V, 298 R/G, 308–310 RIQ/RPELIPVQ, 313 P/S, 314 G/W, ΔV1/V2/V3, ΔV3, 356 N/I, 368 D/R, 368 D/T, 370 E/R, 370 E/Q, 380 G/F, 381 E/P, 382 F/L, 384 Y/E, 386 N/Q, 392 N/E + 397 N/E, 395 W/S, 406 N/G, 420 I/R, 421 K/L, 427 W/V, 427 W/S, 429 K/L, 430 V/S, 432 K/A, 433 A/L, 435 Y/H, 435 Y/S, 438 P/R, 450 T/N, 456 R/K, 457 D/A, 457 D/R, 463 N/D, 470 P/L, 470 P/G, 475 M/S, 485 K/V, 491 I/F, 493 P/K, and 495 G/K.

by some substitutions in the C5 domain (Table 1). These C1-reactive MAbs are exemplified by 4A7C6 (peptide epitope residues 84 to 89), T9 (residues 94 to 100), 133/192 (residues 92 to 96), and 135/9 (108 to 113), but we observed similar results with several other MAbs to the same or closely related epitopes (see below). In contrast, MAb M90 was insensitive to almost all of the amino acid substitutions in C1 and C5 and provides a specificity control (Table 1). This MAb recognizes a discontinuous gp120 epitope (8) that cross-competition studies indicate to be centered on the amino-terminal segment of the C1 domain (data not shown).

The most strongly enhancing amino acid substitutions for all four C1 peptide-reactive MAbs were at residues 256 and 262 (256 S/Y and 262 N/T) (substitutions are indicated as *n* [position] *A* [original residue in single-letter code]/*B* [substituted residue in single-letter code]) in C2, while substitutions at nearby residues 257, 266, and 269 (but not 267) also generally caused enhancement of MAb binding, albeit less strongly. The identity of altered residues can also be an important factor; different substitutions at residue 257 were not equipotent (Table 1). In the C5 domain, changes at residues 475, 485, and 491 usually created up mutants for the C1 peptide-reactive MAbs, as did a single distant-site substitution in C1 at residue 45 (Table 1). Other substitutions closer to the peptide epitopes for the four MAbs had sporadic up or down effects. Indeed, the same amino acid change (88 N/P or 117 K/W) could sometimes cause either an enhancement or diminution of MAb binding. In the case of MAb 4A7C6, the 88 N/P substitution lies within the peptide epitope (residues 84 to 89), probably accounting for the failure of this MAb to bind to that gp120 mutant. It is also notable that substitutions at residues 380, 420, 421, and 427 had weak enhancing or inhibitory effects on the binding of MAbs 4A7C6 and/or 133/192; these three residues lie near the base of the V4 loop and may be topologically adjacent to each other in the folded gp120 molecule. However, the effects of these changes are fairly weak, and their biological significance is uncertain.

As the binding of several C1-reactive MAbs, including those described in Table 1, is increased by denaturation of gp120 (25), we were concerned that the 256 S/Y and 262 N/T substitutions might simply be disrupting the conformation of gp120 to such an extent that the molecule became effectively denatured. However, this seems not to be the case; many HuMAbs to discontinuous, conformationally sensitive epitopes bind the 256 S/Y and 262 N/T mutant gp120s with unchanged, modestly reduced, or even increased efficiency compared with their binding to wild-type HxBc2 gp120. This is exemplified by HuMAbs F105 (38), 15e (36), 48d, and 17b (37). In addition, the 256 S/Y and 262 N/T substitutions do not significantly impair MAb recognition of conformationally sensitive epitopes in the V2 (26) and V3 or C4 (27) regions of gp120 or the discontinuous M90 epitope (Table 1).

A replication-inactivating mutation in C2 is compensated for structurally by a reversion mutation in C1. It has been reported that experimental deletion of an N-linked glycosylation site in the C2 region of the NL4/3 molecular clone of HIV-1 LAI significantly reduced viral replication but that during culture, a revertant virus with nearly wild-type replication kinetics arose (43). This revertant contained a single amino acid substitution in C1, in the conserved stem of the V1-V2 stem-loop structure, which accounted for the phenotypic change (43). Additional analyses of revertant and mutant viruses showed that a single amino acid change in the V3 region of gp120 could also partially compensate for the 267 N/Q mutation (42). In the NL4/3 numbering system, the inactivating substitution was at codon 267 (267 N/Q), while the revertant changes occurred at residue 128 (128 S/N) in C1 or at residue 308 (308 R/I) in V3. Because of minor sequence changes between the NL4/3 and HxBc2 clones, the HxBc2 residues corresponding to NL4/3 amino acids 267 and 308 are numbered 262 and 304, respectively, but position 128 is numbered identically in the two clones. We noted that the inactivating 267 N/Q substitution in NL4/3 therefore involved the same residue that was changed in the HxBc2 262 N/T mutant and that both changes caused the loss of a potential N-linked carbohydrate moiety. In view of the effects of the 262 N/T change on MAb epitope exposure in the C1 region of HxBc2 gp120 (Table 1), we investigated whether the 267 N/Q substitution in NL4/3 gp120 had a similar effect. We also attempted to determine how specific amino acid changes at

TABLE 2. NL4-3 mutants containing specific amino acid substitutions in C1, C2, and V3 of gp120

Mutant designation	Substitution at gp120 codon:		
	128	267	308
NL4-3 (wild type)	S	N	R
128 S/Q	Q	N	R
128 S/N	N	N	R
267 N/Q	S	Q	R
308 R/I	S	N	I
128 S/N + 308 R/I	N	N	I
267 N/Q + 128 S/N	N	Q	R
267 N/Q + 128 S/Q	Q	Q	R
267 N/Q + 308 R/I	S	Q	I

residues 128 and 308, in combination with the 267 N/Q change, affected the conformation of gp120, as measured by antibody binding to gp120.

As recombinant gp120 molecules from the various mutant forms of NL4/3 were not available, we used a different procedure in which cultures of proviral plasmid clones of the wild type HIV-1 NL4/3 and mutant viruses containing defined amino acid changes at gp120 codons 128, 267, and 308 (Table 2) were used as gp120 sources for antigen capture ELISA. To ensure that approximately equal amounts of the different gp120s were captured onto the solid phase, we first titrated the culture supernatants and detected captured gp120 with a pool of HIV-1^+ sera (data not shown). The appropriate amounts of supernatant (1:3 to 1:15 dilutions of the different supernatants) were then used in subsequent experiments. The titration curves for the HIV-1^+ sera pool were essentially superimposable for the different NL4/3 mutants (Fig. 1a).

Previous studies have indicated that amino acid substitutions at residue 267 of NL4/3 of NL4/3 or 262 of HxBc2 could reduce the ability of gp120 to bind sCD4 (31, 43). To explore what effect the 267 N/Q change and the reversion substitutions had on the ability of NL4/3 gp120 to bind sCD4, we performed sCD4 titrations. Two distinct types of binding curve were observed; the 267 N/Q mutant had an approximately 30-fold reduction in sCD4 binding compared with the wild-type gp120 (Fig. 1b), and double mutants containing the 267 N/Q substitution (267 N/Q + 128 S/Q and 267 N/Q + 308 R/I) also had a reduction in sCD4 binding (Fig. 1b). However, gp120 from the reversion mutant (267 N/Q + 128 S/N) bound sCD4 indistinguishably from the wild-type NL4/3 gp120 (Fig. 1b). Thus, the 267 N/Q change has damaged the conformation of the CD4-binding site on gp120, but the 128 S/N reversion has compensated for this defect and restored the CD4-binding site to a configuration able to bind CD4 to an extent comparable with that of the NL4/3 parental gp120. Neither the 128 S/Q change nor the 308 R/I substitution had this compensatory effect for the 267 N/Q change.

We next assessed the effects of these mutants on MAb reactivity with the C1 domain (Fig. 2). Preliminary experiments suggested that a range of C1-reactive MAbs bound more avidly to the 267 N/Q mutant than to the wild-type gp120 or the reversion mutant, 267 N/Q + 128 S/N. Titration curves for two of these MAbs are shown in Fig. 2. Both MAbs T9 (residues 94 to 100) and 135/9 (residues 108 to 113) bound much better to the 267 N/Q, 267 N/Q + 128 S/Q, and 267 N/Q + 308 R/I mutant gp120s than to the wild-type NL4/3 gp120, the 267 N/Q + 128 S/N revertant, or the mutants with amino acid changes only at codon 128 or 308 (Fig. 2). Note that the increased reactivity of the C1 MAbs with the three 267 N/Q-containing mutants (Fig. 2) is the converse of what was seen when sCD4 binding was measured (Fig. 1b). Several other C1-reactive MAbs that recognized two distinct epitope clusters also showed increased binding to the 267 N/Q, 267 N/Q + 128 S/Q, and 267 N/Q + 308 R/I mutant gp120s. These MAbs were 4A7C6 (residues 84 to 89), 133/192 (residues 92 to 96), T7.1 (residues 92 to 97), and B2 (residues 94 to 97) (cluster 1) and 187.2.1 (residues 106 to 112), T2.1 (residues 109 to 113), and M96 (residues 109 to 113) (cluster 2) (data not shown). However, the binding of MAbs M85, M92, 4D4#85, 133/290, and 133/237 to several regions of the C1 domain amino terminal to residue 80 was not significantly affected by the 267 N/Q substitution, exemplified by MAb 4D4#85 to residues 45 to 55 (Fig. 3a). Furthermore, MAb 6D5 to an epitope within residues 122 to 130 failed to bind strongly to any of the gp120s tested (data not shown). This result suggests that the effect of

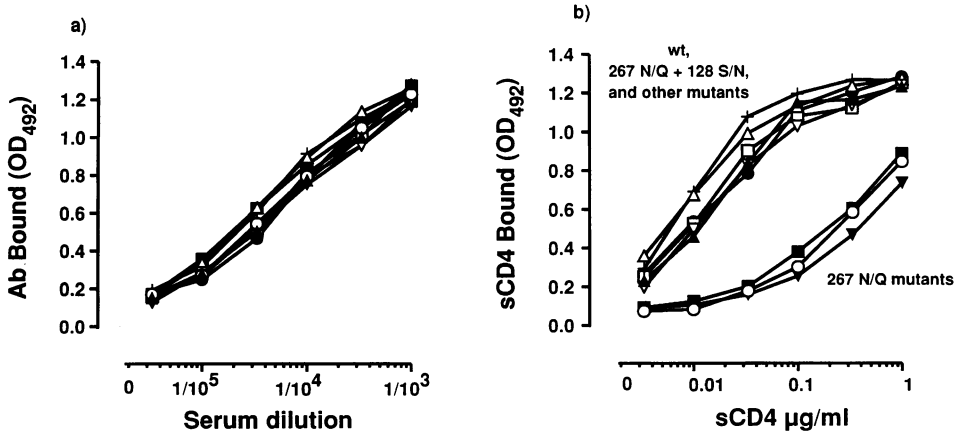

FIG. 1. Reactivities of HIV-1^+ sera and sCD4 with NL4/3 gp120 mutants. Proviral plasmid clones were transfected into HeLa cells, and cell-free culture supernatants were harvested 48 h later to provide sources of the different gp120s. The supernatants were inactivated with nonionic detergent (1% NP-40) and used in antigen capture ELISA. A pool of HIV-1^+ sera (a) or sCD4 (b) at the concentrations indicated was reacted with the various gp120s, and bound human antibodies or sCD4 was detected. The clones tested were NL4/3 (●), 128 S/Q (□), 128 S/N (+), 267 N/Q (■), 308 R/I (△), 267 N/Q + 128 S/Q (▼), 128 S/N + 308 R/I (◆), 267 N/Q + 308 R/I (○), and 267 N/Q + 128 S/N (▲). wt, wild type.

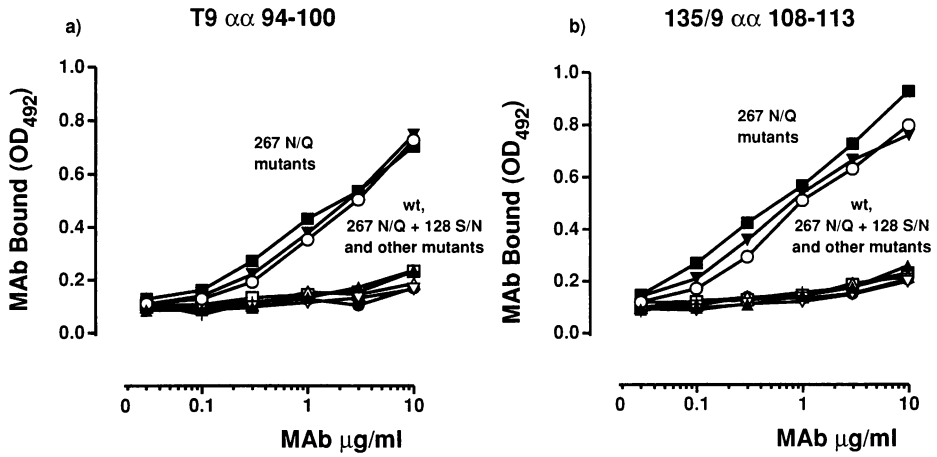

FIG. 2. Reactivities of C1 MAbs with NL4/3 gp120 mutants. MAb T9 (a) or 135/9 (b) at the concentrations indicated was reacted with the various gp120s, and bound MAb was detected. The gp120s tested were NL4/3 (●), 128 S/Q (□), 128 S/N (+), 267 N/Q (■), 308 R/I (△), 267 N/Q + 128 S/Q (▼), 128 S/N + 308 R/I (◆), 267 N/Q + 308 R/I (○), and 267 N/Q + 128 S/N (▲). αα, amino acids; wt, wild type.

the 267N/Q substitution on the structure of the C1 domain is localized to within approximately residues 80 to 120.

It seemed possible that the 267 N/Q substitution could have a gross effect on gp120 structure and that the large increase in the binding of certain C1-reactive MAbs is not restricted to these MAbs alone. We therefore used an extensive panel of MAbs to continuous gp120 epitopes outside the C1 domain to explore the limits of the perturbation of gp120 structure induced by the 267 N/Q substitution. HuMAbs 15e and 21h to discontinuous epitopes bound to both the wild-type NL4/3 gp120 and to the 267 N/Q mutant with comparable affinities (data not shown), indicating that the mutant protein (like the HxBc2 262 N/T mutant) was not merely denatured. Furthermore, with one exception, no MAb to any continuous epitope in the C2, V3, C3, V4, or C4 domain bound significantly more strongly to the 267 N/Q mutants than to NL4/3 gp120 or mutants with other substitutions (summarized in Fig. 4). The single exception was MAb 110.C to an epitope within C2 residues 272 to 279 (HxBc2 numbering); this MAb bound more strongly to the 267 N/Q, 267 N/Q + 128 S/Q, and 267 N/Q + 308 R/I mutants than to the other gp120s, although its binding was always weak (data not shown). Either the epitopes for the others MAbs tested were inaccessible on NP-40-treated gp120 or else their accessibility was not significantly increased by the 267 N/Q substitution (Fig. 4). However, the C5-reactive MAb 1C1 (residues 475 to 485) did show increased binding to the same three 267N/Q-containing mutants that reacted more strongly with some C1 MAbs (Fig. 3b), and a similar, although much weaker, effect was observed with another C5 MAb, CRA-1 (residues 465 to 475) (data not shown). Other V5- or C5-reactive MAbs tested (M91, 9301, and 221) failed to react significantly with any of the NL4/3-derived gp120s, probably because of their low affinity for gp120 that has not been denatured with sodium dodecyl sulfate and dithiothreitol (25). The overall pattern of reactivity of MAbs to continuous gp120 epitopes on the 267 N/Q NL4/3 mutant is summarized in Fig. 4. We conclude that the conformationally disruptive effect of the 267 N/Q

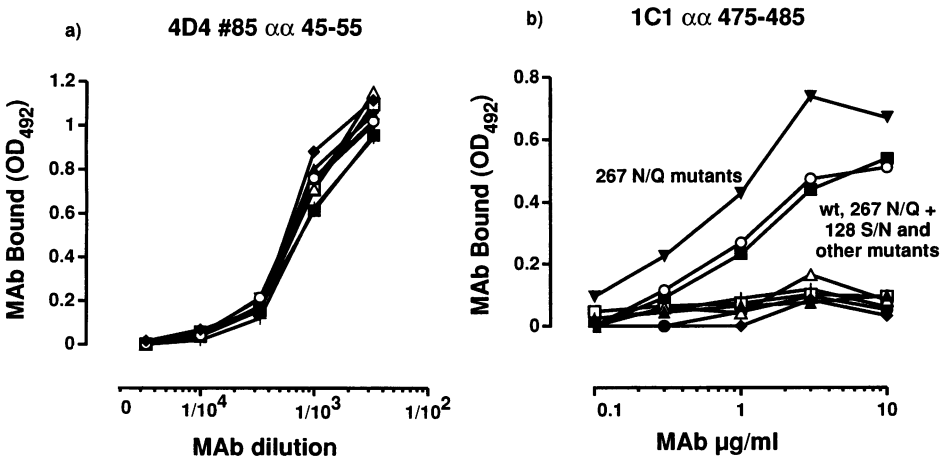

FIG. 3. Reactivities of C1 and C5 MAbs with NL4/3 gp120 mutants. MAb 4D4≠85 (a) or 1C1 (b) at the concentrations indicated was reacted with gp120s, and bound MAb was detected. The gp120s tested were NL4/3 (●), 128 S/Q (□), 128 S/N (+), 267 N/Q (■), 308 R/I (△), 267 N/Q + 128 S/Q (▼), 128 S/N + 308 R/I (◆), 267 N/Q + 308 R/I (○), and 267 N/Q + 128 S/N (▲). The data are corrected for background by subtracting OD_{492} values for antibody binding in the absence of added gp120. αα, amino acids; wt, wild type.

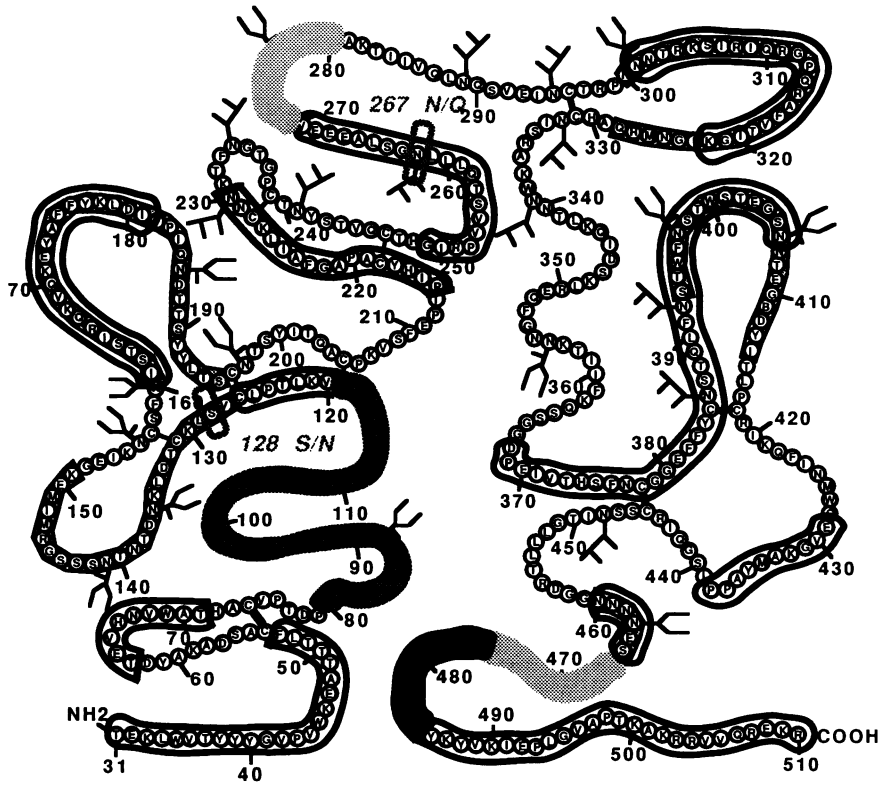

FIG. 4. Summary of continuous regions of gp120 abnormally exposed by the 267N/Q substitution. Depicted on the disulfide map of LAI gp120 (17) are the approximate locations of continuous antibody epitopes. Regions of gp120 unaffected, weakly affected, or strongly affected by the 267 N/Q substitution are indicated by open, hatched, or solid boxes, respectively. The MAbs tested were, reading from the amino terminus of gp120, M85, M92, 4D4#85, 133/237, 133/290, 4A7C6, 133/192, B2, T7.1, T9, T2.1, M96, 187.2.1, 135/9, 6D5, RSD-33, BAT-085, 322-151, J1, MF169.1, MF170.1, M89, B13, 110.E, 110.C, 9284, 110.5, 110.I, C12, B15, G3-299, G45-60, M91, CRA-1, 9301, 1C1, 221, RV110026, and D7324. The epitopes for these MAbs are described elsewhere (25–27).

substitution is mostly restricted to the C1 and C5 domains of gp120.

Functional effects of the C1, C2, and V3 substitutions in NL4/3 gp120. Analyses of the 267 N/Q mutant of NL4/3 and the 267 N/Q + 128 S/N and 267 N/Q + 308 R/I revertant viruses showed that the 128 S/N and 308 R/I amino acid substitutions could both functionally compensate, at least in part, for the deleterious effects of the 267 N/Q change on virus replication (40–43). However, the 308 R/I substitution did not compensate for the 267 N/Q change in terms of sCD4 binding or C1 epitope exposure on monomeric gp120, whereas the 128 S/N change did (Fig. 1 to 3). These findings suggested that the 128 S/N and 308 R/I reversions were acting by different mechanisms. To eliminate the possibility that this difference was simply attributable to the use of an assay system measuring sCD4 binding to detergent-solubilized, monomeric gp120, we examined envelope function in the context of the native, oligomeric protein found on virions. Using an assay able to monitor the kinetics of sCD4 binding to virions (44), we found that the rates of binding of CD4 IgG to the wild type and the 267 N/Q + 128 S/N mutant were virtually identical (Fig. 5), whereas CD4 IgG binding to the 267 N/Q + 308 R/I mutant was much slower, irrespective of whether the CD4 IgG concentration used was 50 nM (Fig. 5A and B) or 5 nM (Fig. 5C). The data from these studies with oligomeric envelope glycoproteins are therefore consistent with those derived from monomeric gp120 (Fig. 1b); therefore, we conclude that the 128 S/N and 308 R/I changes differentially affect gp120 structure and function in the context of the 267 N/Q substitution, while both compensate for the replication-reducing effect(s) of the latter change.

Evidence for interactions between the C1, C2, and C5 domains. The influence of C2 substitutions on the binding of MAbs to the C5 domain that we described above (Fig. 3b) was confirmed by using the panel of HxBc2 mutants in the absence of detergent. Several substitutions in C2, including the 262 N/T change, increased the binding of five MAbs to three separate continuous epitopes within residues 460 to 485 (Table 3). However, the magnitude of the enhancing effects and the number of C2 changes that caused enhancement were less for the C5-reactive MAbs than was observed with the C1-reactive MAbs (Table 1). Other than inhibitory or enhancing substitutions in C5 close to the location of the continuous epitope, changes elsewhere in gp120 influencing C5-MAb binding were rare (Table 3); several changes involving the V1, V2, and V3 loops had weak, sporadic inhibitory or enhancing effects, the significance of which is as yet unclear.

The results presented above suggest that the disruption of gp120 conformation caused by amino acid changes in C2 extends to the C5 domain as well as the C1 domain. In principle, the effect on C5 structure could be mediated directly, if amino acids in C2 impinged directly on those in C5, or indirectly, if a C2-mediated perturbation of C1 conformation were transmitted into the C5 domain via a contact between C1 and C5. While we cannot unequivocally distinguish between these possibilities, studies with MAbs do suggest that there is

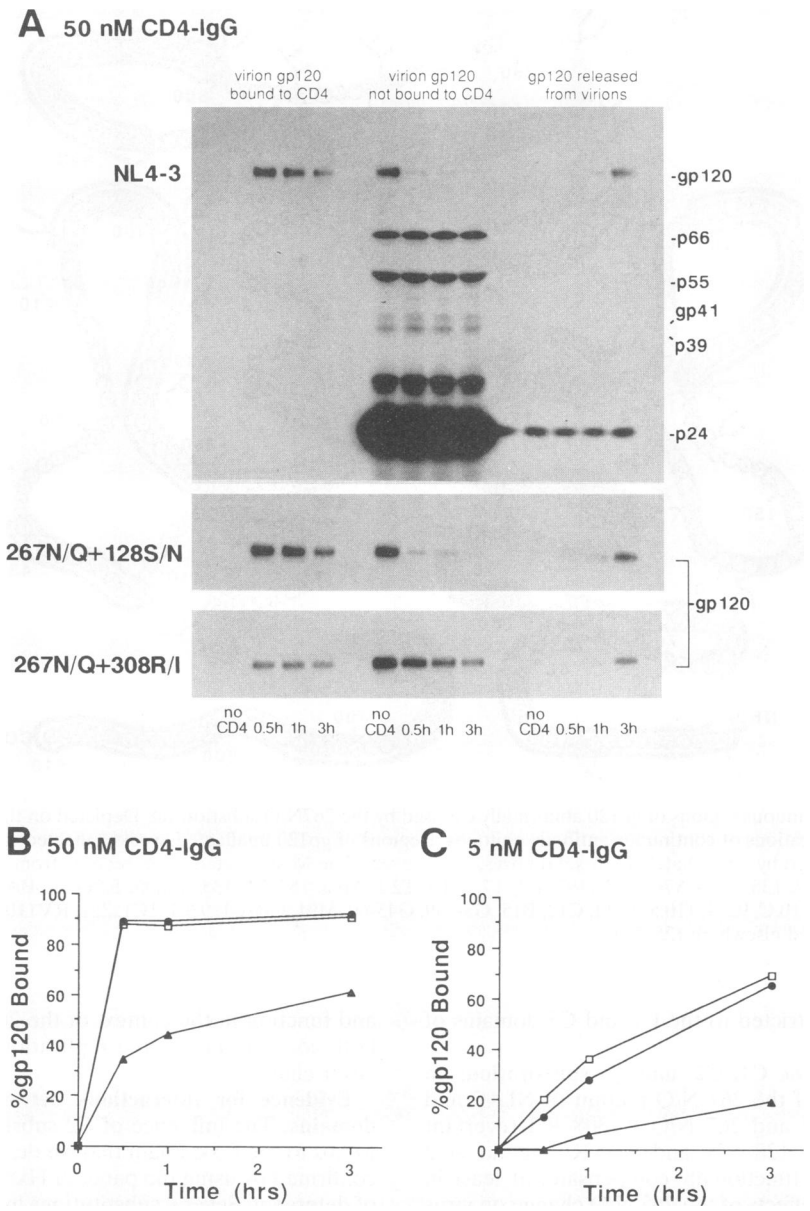

FIG. 5. Binding of CD4 IgG to NL4/3 and mutant virions. (A) ^{35}S-labeled virions were incubated with 50 nM CD4 IgG for 0.5, 1, and 3 h; the amounts of virion-associated gp120 bound and not bound to CD4 IgG and the amounts of gp120 released from the virions were determined and are shown. (B) Data derived from panel A by Phosphoimager quantitation of the autoradiograms are represented in graphical form. (C) As panels A and B except that the CD4 IgG concentration was reduced to 5 nM. The viruses used were NL4/3 (□), 267 N/Q + 128 S/N (●), and 267 N/Q + 308 R/I (▲).

an association between the C1 and C5 domains (Tables 1 and 4). First, amino acid substitutions in C5 at residues 475, 485, and 491 tended to increase the binding of MAbs to continuous epitopes within residues 80 to 113 of the C1 domain (Table 1). Second, two nonneutralizing HuMAbs recognizing conformationally sensitive or discontinuous epitopes were tested for reactivity with the panel of HxBc2 gp120 mutants. The binding of HuMAb C11 was abolished, or very strongly impaired, by amino acid substitutions in both the C1 (45 W/S and 88 N/P) and C5 (491 I/E, 493 P/K, and 495 G/K) domains and was weakly inhibited by the 463 N/D substitution in V5. Another C1 change (36 V/L) increased C11 binding (Table 4). A second HuMAb, 212A, was very sensitive to the 45 W/S change, and its binding to the 463 N/D mutant was reduced. Conversely, the 495 G/K substitution caused a strong enhancement of 212A binding (Table 4). As observed with some of the MAbs to continuous C5 epitopes, a few amino acid substitutions in the V1-V2 loops, or in C1 at the base of this of this loop structure, caused a modest enhancement of C11 and 212A binding (Table 4), the significance of which is uncertain at present.

To confirm that segments of both the C1 and C5 domains were proximal to the epitope for MAb C11, we performed cross-competition studies (Fig. 6). The binding of C11 to BH10 gp120 was inhibited to various extents by MAbs reactive with continuous epitopes in the C1 and C5 domains. Thus, MAbs M85 (residues 30 to 51) and M91 (residues 461 to 470) were

TABLE 3. Amino acid substitutions influencing the binding of MAbs to continuous epitopes in the C5 domain of gp120

Mutant (domain)	Normalized binding ratio[a]				
	M91	CRA-1	660-178	221	1C1
Down					
313 P/S (V3)	0.88	0.61	0.68	**0.42**	**0.49**
314 G/W (V3)	0.87	**0.48**	0.68	**0.39**	0.53
ΔV1/V2	0.54	0.52	**0.40**	**0.37**	**0.41**
ΔV1/V2/V3	0.75	0.82	**0.49**	0.76	0.57
470 P/L (C5)	**0.01**	**0.00**	0.85	0.89	0.84
470 P/G (C5)	**0.09**	**0.00**	1.36	1.26	1.35
475 M/S (C5)	1.18	**0.00**	1.40	0.97	1.20
477 D/V (C5)	0.60	**0.42**	1.82	**0.00**	0.90
485 K/V (C5)	1.15	**0.48**	0.92	0.55	0.53
Up					
152/153 GE/SM (V1/V2)	0.99	1.33	0.93	**1.55**	1.02
176/177 FY/AT (V2)	1.25	**1.58**	0.86	1.24	1.20
256 S/Y (C2)	**2.06**	**1.79**	**1.78**	**2.47**	**2.16**
257 T/R (C2)	1.49	1.24	1.11	1.26	1.29
257 T/A (C2)	1.45	1.48	1.36	**1.84**	**1.71**
257 T/G (C2)	0.69	0.61	0.73	0.82	1.04
262 N/T (C2)	**2.81**	**2.55**	**2.58**	**1.58**	**2.20**
269 E/L (C2)	1.19	0.94	1.12	**1.58**	1.18
457 D/R (C5)	1.04	1.42	1.25	**1.97**	1.12
477 D/V (C5)	0.60	0.42	**1.82**	0.00	0.90

[a] The peptide epitope residues to which the MAbs bound and the mean binding ratios ± SD (normalized to a value of 1; see Materials and Methods) are as follows: M91, 461 to 470 and 0.67 ± 0.14; CRA-1, 470 to 480 and 0.33 ± 0.10; 660-178, 475 to 485 and 0.73 ± 0.22; 221, 475 to 485 and 0.38 ± 0.16; 1C1, 475 to 485 and 0.49 ± 0.15. MAbs were tested at the following concentrations: M91, 1:1,000 dilution of ascites fluid; CRA-1, 1:10,000 dilution of ascites fluid; 660-178, 0.5 μg/ml; 221, 3 μg/ml; 1C1, 0.03 μg/ml. The panel of HxBc2 gp120 mutants was the same as for Table 1 except that the 495 G/K mutant was replaced by the 477 D/V mutant. Boldface values are as described for Table 1.

TABLE 4. Amino acid substitutions influencing the binding of HuMAbs to discontinuous epitopes in the C1 and C5 domains of gp120[a]

Mutant (domain)	Normalized binding ratio	
	C11 (0.51 ± 0.13[b])	212A (0.82 ± 0.21)
Down		
45 W/S (C1)	**0.16**	**0.07**
88 N/P (C1)	**0.20**	0.87
463 N/D (V5)	**0.47**	**0.45**
491 I/F (C5)	**0.04**	1.05
493 P/K (C5)	**0.00**	1.20
495 G/K (C5)	**0.04**	1.72
Up		
36 V/L (C1)	**1.65**	1.34
80 N/R (C1)	1.45	1.13
120/121 VK/LE (C1)	1.07	1.48
125 L/G (C1)	1.43	1.43
152/153 GE/SM (V1/V2)	**1.55**	1.32
179/180 LD/DL (V2)	1.45	**1.51**
ΔV1/V2/V3	**1.57**	1.32
495 G/K (C5)	0.04	**1.72**

[a] MAbs were tested at the following concentrations: C11, 10 μg/ml; 212A, 5 μg/ml. The panel of HxBc2 gp120 mutants and boldface values are as described for Table 1.

[b] Mean binding ratio ± SD (normalized to a value of 1; see Materials and Methods).

moderately strong inhibitors of C11 binding, while MAbs 4D4#85 (residues 41 to 50) and CRA-1 (residues 465 to 475) had weaker inhibitory effects (Fig. 6). The combination of M85 and M91 caused almost complete inhibition of C11 binding (data not shown). In addition, MAbs C11 and 212A were mutually cross-competitive in their binding to gp120, and the binding of either MAb was completely blocked by MAbs M90 and MAG-45, which recognize discontinuous epitopes centered on the C1 domain (data not shown). In contrast, the C1 MAbs 133/290 and 4A7C6, the V2 MAb 684-238, the V3 MAbs 110.5 and 110.I, the CD4-binding site MAbs 21h and MAG-55, and CD4 IgG had no significant inhibitory effect (<25% reduction) on the binding of MAb C11 to gp120 (data not shown). Taken together with the susceptibility of C11 to amino acid substitutions in both the C1 and C5 domains (Table 4), the cross-competition data strongly suggest that the epitope for MAb C11 incorporates C1 and C5 residues. This implies that these regions of gp120 are proximal in the folded protein.

DISCUSSION

The HIV-1 gp120 envelope glycoprotein is a large, complex molecule, and little is known of its secondary and tertiary structure. On the basis of genetic sequence analysis, conserved and variable regions of the protein have been identified (17, 21, 29, 34). We have previously shown that a significant proportion of the variable regions of HxBc2 gp120 is antibody accessible in the context of the monomeric form of the protein, whereas the conserved regions tend to be antibody inaccessible, especially on the native, gp120/gp41 oligomer (25). It is reasonable to assume that the conserved regions of gp120 are located in the interior of the molecule or else involved in contacting other components of the oligomer (25). This conclusion is supported by the generally hydrophobic nature of the conserved domains. However, we lack information as to how the different domains pack together in the folded protein.

A number of genetic and immunological analyses have indicated that there can be functional interdomain interactions in gp120. The first demonstration of this was the observation that a virus-inactivating amino acid substitution in the C2 region was compensated for by a reversion substitution in the C1 domain, at a site distantly located in the primary sequence (42, 43). This finding implied that the C1 and C2 domains must interact in some way, and the exploration of this notion is one of the foci of our present study. Other studies have indicated that the amino-terminal flank of the V3 loop interacts in some way both with the C1-C2 structure (42) and with the C4 region (27, 28, 46), while there is also evidence for an interaction between the carboxy-terminal side of the V3 loop and a segment of C2 around amino acid 281 (5). Furthermore, the V2 and C4 regions of gp120 also appear to be related spatially to one another (12, 26).

In this study, we focus on the C1, C2, and C5 domains and supply a structural explanation for the genetic observations of Willey et al. (42, 43) that suggested a functional interaction between the C2 region near asparagine 267 of NL4/3 and the C1 region near serine 128. The 267 N/Q substitution alters the conformation of a large segment of the C1 domain spanning approximately residues 80 to 120 in such a way that it becomes more antibody accessible. A stretch of the C2 domain represented by the MAb 110.C epitope between residues 271 and 280 also becomes more exposed. These conformational changes, en passant, compromise the integrity of the CD4-binding site, causing an at least 30-fold reduction in sCD4 binding. These rather dramatic changes in gp120 conformation fall short of denaturation, since several antibody epitopes that have been shown to be discontinuous remain intact on the NL4/3 267 N/Q mutant and on the similar HxBc2 mutants 256 S/Y and 262 N/T (36–38).

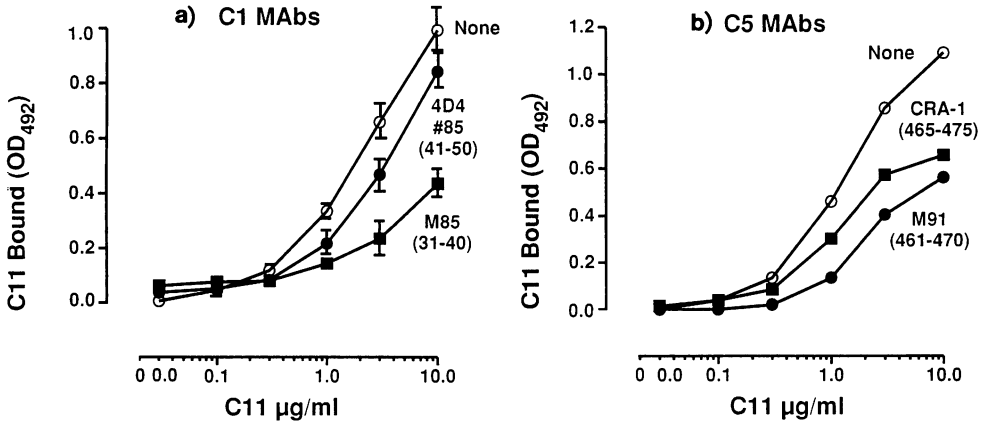

FIG. 6. Competition for HuMAb C11 binding to gp120 by C1 and C5 MAbs. HuMAb C11 was incubated at the concentrations indicated with BH10 gp120 in the presence of no competitor, 4D4≠85, or M85 (■) (a) or no competitor, M91, or CRA-1 (b), and bound C11 was detected. The competitor MAbs were added as 1:100 dilutions of ascites fluid for 30 min prior to the addition of C11. The datum points represent means ± SDs of triplicate wells (a) or means of duplicate wells (b). Assay background (no C11 added) was subtracted from each datum point.

The reduced CD4-binding ability alone might be sufficient to account for the decreased replication competence of the 267 N/Q mutant if the perturbation of the CD4-binding site were the only effect of the 267 N/Q substitution. This could account for, at least in part, the delayed infection kinetics of the 267 N/Q + 308 R/I virus. However, the 267 N/Q substitution also causes a reduction in gp120 association with virions (40), perhaps because of a disruption of the gp41-binding region in the C1 and C5 domains (15). This effect is most likely a major contributing factor to the replication incompetence of the 267 N/Q mutant (41). The reversion mutation, 128 S/N, functionally compensates for the effect of the 267 N/Q mutation by restoring the correct conformation of the C1 domain, thereby re-creating not only the CD4-binding site (Fig. 1b and 5) but also the ability of gp120 to remain associated with virions (40). A different change at residue 128 (128 S/Q) is unable to compensate for the 267 N/Q substitution either functionally (42) or in terms of restoring gp120 conformation, demonstrating the specificity of the effect.

It is important to note that while the 128 S/N change appears to compensate completely for the 267 N/Q substitution in the assays of gp120 conformation used in this study, the infectivity of the 128 S/N revertant has not been fully restored to wild-type levels (42, 43). Furthermore, the 308 R/I substitution can also compensate for the 267 N/Q substitution by restoring the infectivity of NL4/3 to near wild-type levels (42). The 308 R/I change, like the 128 S/N substitution, increases the amount of gp120 in association with virions that is otherwise low in the presence of the 267 N/Q substitution (41). However, the 308 R/I change does not compensate for the 267 N/Q change in the CD4-binding assays used in this study (Fig. 1b and 5). Thus, the effect of the 267 N/Q change on gp120 folding may affect envelope function and virus infectivity more significantly than suggested by the conformational changes documented herein.

What is the nature of the C1-C2 association? Secondary structural predictions indicate that the C1 segment spanning residues 82 to 117 forms an amphipathic alpha helix (Fig. 7a) (13). Within that helix are two immunodominant epitopes, residues 91 to 99 and 104 to 112, recognized by numerous C1 MAbs, including several used in this study, and a separate epitope for MAb 4A7C6 (1, 18, 25). We presume that the entire alpha helix becomes abnormally antibody accessible as a consequence of the substitution of threonine for asparagine at residue 262 in the C2 domain (or glutamine for asparagine at residue 267 of NL4/3). Asparagine 262 is also predicted by folding algorithms to lie within an amphipathic alpha helix (Fig. 7b) (13). Our previous study of the accessibility of linear gp120 epitopes indicates that neither of the predicted C1 and C2 helices is well exposed on the native gp120 glycoprotein monomer (25). The attached carbohydrate at asparagine 262 maintains a high-mannose structure (17), consistent with this residue being inaccessible for complex sugar addition after a certain point in the process of envelope glycoprotein folding, as discussed elsewhere (25). The penultimate residue of the predicted C2 alpha helix (asparagine 276) is another canonical N-linked glycosylation site; however, the carbohydrates on asparagine 276 are complex (17), perhaps indicating that this region of the alpha helix remains accessible on a more mature product of the glycoprotein-folding pathway.

The data provided in this report support a model in which the amphipathic alpha helices in the C1 and C2 domains are located in proximity in the folded gp120 protein. The relationship between the C1 and C2 domains may be disrupted by the 262 N/T (267 N/Q) substitution and restored by the reversion substitution 128 S/N. Residue 128 lies outside the predicted alpha helix in C1, within the conserved stem of the V1-V2 stem-loop structure (17). The ability of an amino acid substitution at this residue to restore the C1-C2 relationship suggests that the conserved V1-V2 stem may be folded in proximity to parts of the predicted C1-C2 helices.

Taken together, the MAb-mapping and cross-competition data also suggest that there is an association between the C1 and C5 domains of gp120 within the hydrophobic core of the molecule and that this interaction is perturbed by amino acid substitutions in the C2 domain, notably at residues 256 and 262 (residue 267 of NL4/3). A previous study has shown that several amino acid substitutions in C1 and C5, notably at residues 36, 40, 45, 491, 493, 495, and 497 to 501, disrupt the association between gp120 and gp41 (15). Although other explanations of the genetic data are clearly feasible, one interpretation of these observations is that residues in both C1 and C5 are folded into proximity to form a discontinuous structure involved in gp41 binding. Our new data, notably the genetic and cross-competition analyses of HuMAb C11 binding, are consistent with this interpretation. One possibility is that the epitope for HuMAb C11 spans the C1/C5 junction, in

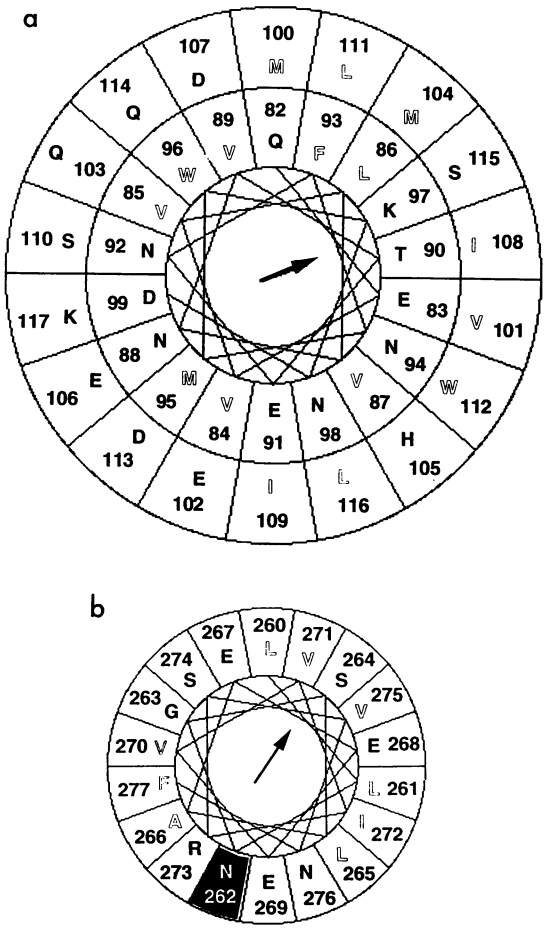

FIG. 7. Secondary structural predictions of the gp120 C1 and C2 domains. Secondary structural predictions were carried out by using the Chou-Fasman (6) and Robson-Garnier (9) algorithms in a combination that predicts an alpha-helical content of 27.9% for gp120. This calculated value agrees well with the observed value of 21.7% obtained by Fourier transform infrared spectroscopy (7). The regions spanning positions 82 to 117 and 260 to 277 were predicted to be alpha helical. These segments include (i) the regions recognized by the C1-specific MAbs whose binding to gp120 is influenced by substitutions in the C2 domain and (ii) the relevant section of the C2 domain itself. Helical-wheel analyses (33) are depicted for each of the helices to illustrate the predicted orientation of substituted residues on the faces of the helices. The arrows denote the direction of the hydrophobic moment, which is an indication of the more hydrophobic face of the helix (i.e., the face that is least likely to be solvent accessible). The values for hydrophobic moments are 0.71 for the 82–117 region and 0.77 for the 260–277 region. Hydrophobic residues are represented by an outlined font. The helical wheels depicted are derived from the C1 region, residues 82 to 117 (a), and the C2 region, residues 260 to 277 (b). The numbering system is based on the HxBc2 sequence.

which case the two domains would be in sufficient proximity to contribute to an antibody footprint. Consistent with the oft-reported inability of C1 and C5 MAbs to neutralize HIV-1 infectivity (1, 8, 30), HuMAbs C11 and 212A do not neutralize HIV-1 IIIB (32). Furthermore, neither is able to bind to the surface of HIV-1 IIIB-infected cells, on which gp120 is present in its native, oligomeric configuration (data not shown). Presumably, the putative C1 and C1/C5 epitopes for these MAbs, while clearly accessible on the gp120 monomer, are occluded by other components of the oligomeric complex. The immunogens for these human antibodies were probably soluble gp120 molecules released from virions or virus-infected cells.

In conclusion, our studies reinforce our picture of gp120 as a complex, precisely folded molecule to which the conserved, hydrophobic domains contribute the internal structural elements. Interdomain associations stabilize this core structure, and we suggest that one of these associations involves segments of the C1 and C2 domains. An additional interaction between the C1 and C5 domains may create a gp41-binding site (15). Given our observations that continuous antibody epitopes in the C1 and C2 alpha-helical regions, and in the C5 domain, tend to be poorly exposed on the surface of gp120 (18, 25), it seems possible that the C1, C2, and C5 domains all interact within the internal core of gp120. Mutations leading to amino acid substitutions in one domain can have profound effects on the overall conformation of gp120 by impacting on interdomain associations; this complicates the interpretation of how any one mutation might act. For example, the 267 N/Q (262 N/T) substitution not only perturbs CD4 binding (Fig. 1) but also affects the association of gp120 with gp41 (15, 41). This could be mediated via a direct effect of the 267 N/Q substitution on the conformation of the gp41-binding site. Alternatively, the disruption of the conformation of the C1 domain caused by the amino acid change in C2 might influence indirectly the formation of a gp41-binding site formed predominantly by C1 and C5 residues. A more complete understanding of the relationship between gp120 conformation and its function will require additional genetic, immunological, and structural analyses.

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