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Immunochemical Analysis of the gp120 Surface Glycoprotein of Human Immunodeficiency Virus Type 1: Probing the Structure of the C4 and V4 Domains and the Interaction of the C4 Domain with the V3 Loop

JOHN P. MOORE,[1]* MARKUS THALI,[2] BRADFORD A. JAMESON,[3] FRANÇOISE VIGNAUX,[4] GEORGE K. LEWIS,[5] SHU-WING POON,[1] MACARTHUR CHARLES,[2] MICHAEL S. FUNG,[6] BILL SUN,[6] PAUL J. DURDA,[7] LENNART ÅKERBLOM,[8] BRITTA WAHREN,[8,9] DAVID D. HO,[1] QUENTIN J. SATTENTAU,[4] AND JOSEPH SODROSKI[2]

Aaron Diamond AIDS Research Center, New York University School of Medicine, New York, New York 10016[1]; Division of Human Retrovirology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115[2]; Jefferson Cancer Institute, Philadelphia, Pennsylvania 19107[3]; Centre d'Immunologie de Marseille-Luminy, Marseille 13288, France[4]; University of Maryland Medical School, Baltimore, Maryland 21201[5]; Tanox Biosystems, Houston, Texas 77025[6]; Genzyme Corporation, Cambridge, Massachusetts 02139[7]; Veterinary Virology, Biomedical Center, Uppsala, Sweden[8]; and National Bacteriological Laboratory, Karolinska Institute, Stockholm 10521, Sweden[9]

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We have probed the structure of the C4 and V3 domains of human immunodeficiency virus type 1 gp120 by immunochemical techniques. Monoclonal antibodies (MAbs) recognizing an exposed gp120 sequence, (E/K)VGKAMYAPP, in C4 were differentially sensitive to denaturation of gp120, implying a conformational component to some of the epitopes. The MAbs recognizing conformation-sensitive C4 structures failed to bind to a gp120 mutant with an alteration in the sequence of the V3 loop, and their binding to gp120 was inhibited by both V3 and C4 MAbs. This implies an interaction between the V3 and C4 regions of gp120, which is supported by the observation that the binding of some MAbs to the V3 loop was often enhanced by amino acid changes in and around the C4 region.

The outer envelope glycoprotein gp120 of human immunodeficiency virus type 1 (HIV-1) mediates viral attachment to the cell surface glycoprotein CD4 in the initial phase of HIV-1 infection of $CD4^+$ cells (18, 21). It is relevant to our understanding of the interaction of HIV-1 with its receptor to know the shape of gp120, and it may be important for future vaccine design to know how the domains of gp120 are presented to the human immune system. In the absence of a crystal structure for this extensively glycosylated protein, we are analyzing the conformation of the gp120 glycoprotein by immunochemical techniques. Our strategy includes exploring the structures of individual domains with monoclonal antibodies (MAbs) and assessing how amino acid substitutions in one region of gp120 affect the binding of MAbs to other regions.

The fourth conserved (C4) domain was initially implicated as important for the interaction of gp120 with CD4 when it was observed that deletion of 12 amino acids from this region rendered gp120 unable to bind CD4 (17). Two MAbs against the C4 region also blocked recombinant gp120 from binding CD4 (7, 17). These early observations have led to the designation of a short linear amino acid sequence in C4 as the CD4 binding site. Concurrent studies, however, suggested that amino acid changes outside of the C4 region could also disrupt the CD4-binding ability of gp120 (14). More recent studies support the concept that the CD4-binding site is a structure incorporating amino acids from several regions of gp120 that are widely separated in the linear sequence but are presumably folded into proximity in the native protein (32). Only one of these key amino acids, Trp-427, is in C4 (6, 32), and substitutions of other C4 amino acids are without major effect on CD4 binding (32). Nonetheless, MAbs to this domain can block CD4 binding (5, 7, 17, 22, 31, 37), and amino acid changes in C4 can affect the fusion process critical for virus entry (38), as can changes in the third variable (V3) loop (28, 38). Thus, we wish to understand more fully the structure and function of C4 in the context of the gp120 protein.

It has recently been reported that changes in the amino acid sequence of the C4 domain can increase the binding of an MAb to the amino-terminal side of the V3 loop of gp120 (45). Conversely, some changes in the base of the V3 loop increased recognition of gp120 by antipeptide antibodies directed against C4 (45). Here we provide additional evidence suggesting a structural relationship between the V3 loop and the C4 region: two MAbs are prevented from binding to the C4 domain by a three-amino-acid insertion into the amino-terminal side of the V3 loop; the binding of other C4 MAbs is inhibited by a Gly-Trp substitution at the crown of the V3 loop; and MAbs directed against the V3 loop are able to inhibit the binding of some C4 MAbs to gp120. Taken together, these observations imply that these two domains of gp120 are in proximity in the folded gp120 glycoprotein, which could be important for understanding gp120 conformational changes that occur subsequent to CD4 binding (33).

MATERIALS AND METHODS

MAbs, recombinant gp120s, and peptides. Murine MAbs G3-42, G3-299, G3-508, G3-519, and G3-536 were isolated by

* Corresponding author.

Sun et al. (37), and MAb G45-60 was prepared by a similar procedure. The immunogen was gp120 purified from HIV-1 IIIB-infected cell cultures. Some of these MAbs were biotinylated, as described previously (37). MAb 0.5β (19, 34) was obtained from the National Institute for Allergy and Infectious Disease reagent repository. MAbs 110.4 and 110.5 (13) and 9284 (34) were purchased from Genetic Systems, Inc. (Seattle, Wash.), and Du Pont Inc., respectively. MAbs 5042 43.1 and 5021 22.2 have been described elsewhere (8, 9), as have P1/D12 and P4/D10 (2, 3). MAb F58/D1 is a human version of the murine MAb F58/H3 described previously (2, 3). The production of MAbs B15, B12, and C32 is documented by Abacioglu et al. (1). Rat MAbs ICR 38, ICR 39.3b, and 39.13g (5) were obtained from the AIDS Directed Programme (ADP) Reagent Repository (United Kingdom Medical Research Council), as was murine MAb 36.1 (ADP 329) (42). ICR 38 was originally reported to be two independent MAbs, ICR 38.1a and ICR 38.8f (5), but they are actually subclones of the same MAb. MAbs 110.D and 110.I were gifts from F. Traincard (HybridoLabs, Paris, France). MAb 684-238 to a V2 epitope has been described elsewhere (29). Rabbit antisera R213 to poliovirus chimera S1/env/9 and R229 to chimera S1/env/10 (22) were donated by D. Evans (University of Reading, Reading, United Kingdom).

HIV IIIB gp120 (BH10 clone) expressed in CHO cells by Celltech Ltd. (Slough, United Kingdom) was obtained from the ADP Reagent Repository (27). SF-2 gp120 expressed in CHO cells (35) was a gift from Kathy Steimer and Nancy Haigwood, Chiron Inc. (Emeryville, Calif.). MN gp120 purified from the supernatants of infected cells was provided by Larry Arthur (National Cancer Institute, Frederick, Md.). Mutants of IIIB gp120 (H×B2 clone) with alterations in one or a few amino acids were transiently expressed in COS-1 cells as described previously (10, 11, 32, 36, 38–41). The culture supernatants containing secreted gp120 were stored at −40°C.

A set of 20-mer gp120 peptides (BH10 sequence) overlapping by 10 amino acids was obtained from the ADP reagent repository (ADP 740). A separate set of 20-mer peptides from the H×B2 sequence, also overlapping by 10 amino acids but slightly out of phase with the first set, was purchased from Cambridge Research Biochemicals (Hartston, United Kingdom). Decamer peptides from the C4 region overlapping by two amino acids were obtained from the ADP Reagent Programme (ADP 742) (22). The sequence of these peptides was from HIV-1 BRU. There is an E/K polymorphism between BRU and H×B2 at position 429 in C4. These peptides were dissolved at 500 μg/ml in saline containing 75% dimethyl sulfoxide. Peptides from the V3 regions of H×B2 and SF-2 were purchased from American Biotechnologies, Inc. (Cambridge, Mass.). A cyclized 36-mer peptide corresponding to the BRU V3 region, but with a single Arg for Lys substitution at amino acid 305, was obtained from the ADP Reagent Repository (ADP 737). This peptide was also used after treatment with dithiothreitol (DTT) and iodoacetamide to render it linear. A control peptide from gp41 (amino acids 735 to 752) was also obtained from the ADP Reagent Repository (ADP 707). These latter peptides were all dissolved at 1 to 5 mg/ml in Tris-buffered saline (TBS).

Binding of MAbs to peptides or recombinant monomeric gp120; competition with MAbs; digestion of gp120 with thrombin. The gp120 glycoproteins were captured onto a solid phase via adsorbed antibody D7324 to the carboxy-terminal 15 amino acids, essentially as described previously (24, 27). All immunoassay procedures were carried out at room temperature. For experiments on native gp120, the buffer used was TBS supplemented with 10% fetal calf serum (TBS-FCS). In some experiments, gp120 was denatured by boiling at a 10× concentration for 5 min in TBS-FCS containing 1% sodium dodecyl sulfate (SDS) and 50 mM DTT before dilution 10-fold into TBS-FCS plus 1% Nonidet P-40 (26). MAbs were bound onto gp120 in TMSS buffer (2% nonfat milk powder and 20% sheep serum in TBS), which was supplemented with 0.5% Tween 20 for experiments on denatured gp120. For experiments on normally folded, recombinant gp120, the use of detergents was completely avoided. Bound murine MAb was detected with alkaline phosphatase (AP)-conjugated rabbit anti-mouse immunoglobulin (Ig; Dako Diagnostics), rat MAb with AP-conjugated goat anti-rat IgG/IgM (Accurate Chemicals), and human MAb with AP-conjugated goat anti-human IgG (Accurate Chemicals) and then with the AMPAK amplification system (Dako Diagnostics).

For MAb competition experiments, the competitor MAb was added to D7324-immobilized gp120 for 30 min in 50 μl of TMSS buffer before addition of a fixed concentration of biotin-labeled C4 MAb in a further 50 μl of TMSS buffer. After incubation for 1 h, the amount of biotin-labeled MAb bound to gp120 was determined by using AP-conjugated streptavidin (Dako Diagnostics) and AMPAK.

For peptide competition experiments, peptides at 1 to 5 μg/ml were incubated for 1 h with MAb before addition to solid-phase-adsorbed gp120 and determination of the amount of bound MAb (25). A concentration of each MAb in the range of 3 to 15 nM was chosen to give equivalent gp120 binding.

To cleave the V3 loop of gp120 with thrombin (4, 33), gp120 captured onto D7324 was incubated for 2 h at 37°C with TBS containing 0.5% FCS with or without a 1:20 dilution of bovine thrombin (Boehringer, Mannheim, Germany). The thrombin stock was prepared by addition of 1 ml of TBS to a vial of lyophilized thrombin. After the digestion, thrombin was removed by washing the plates with TBS before MAb addition.

Peptide-binding assays were performed by adsorbing peptides at 10 μg/ml, or as indicated, in TBS onto Immulon II enzyme-linked immunosorbent assay (ELISA) plates (Dynatech Ltd.). After the unbound peptides were washed off and the plates were blocked for 30 min with a solution of 2% nonfat milk powder in TBS, MAbs were added (usually at 1 μg/ml [6.7 nM]) in TMSS buffer plus 0.5% Tween 20 (25). Bound MAbs were detected as outlined above. Some MAbs were also analyzed by pepscan techniques (1).

Mapping epitopes by ELISA with H×B2 gp120 mutants. Culture supernatants (100 μl) from COS-1 cells transfected 48 h previously with 10 μg of plasmid pSVIIIenv expressing either wild-type or mutant HIV-1 (H×Bc2) envelope glycoproteins (10, 11, 32, 36, 38–41) were supplemented with 20 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.0). The gp120 molecules in the supernatants were captured onto a solid phase via antibody D7324 in the absence of detergent, 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 usually selected from prior titration curves to give approximately 75% of the level of binding at saturating MAb concentrations. Optical density at 492 nm (OD_{492}) values were generally in the range from 0.3 to 0.8 for each mutant with most of the MAbs. Because this level of binding is nonsaturating for our detection systems, we were able to seek enhancing as well as inhibitory amino acid substitu-

tions. All the gp120 mutants were accommodated in a single ELISA plate.

We chose to use culture supernatants containing secreted gp120 rather than detergent-solubilized, transfected cells to avoid potential artifacts associated with the capture of incompletely folded gp160 molecules onto the solid phase (23). The concentrations of gp120 in the different culture supernatants vary. Partly for this reason, and partly because normalization to a single well is too susceptible to random or systematic error in an ELISA format, we assessed the significance of our binding data by normalizing to the whole panel of mutants rather than just to wild-type gp120. Thus, we measured, in quadruplicate plates, the reactivities of the gp120 mutants with a pool of HIV-1$^+$ human sera, binding of the serum 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} for each mutant was determined as a reference value. Each test MAb was assessed for binding to the mutant panel in duplicate, or more usually in triplicate, and the mean OD_{492} was determined. For each mutant, the OD_{492} ratio of the test MAb to the reference serum was determined, and the average value of this ratio for the whole panel was then calculated (mean ± standard deviation), omitting values close to zero (amino acid changes that abolish binding) from the calculation to avoid skewing the data. Ratios that deviated the most from the mean value were then highlighted as indicating inhibitory or enhancing changes in the amino acid sequence. Ratios of less than 0.5 times or more than 1.5 times the mean ratio were considered probably significant, although the smaller the deviation of a value from the norm, the more likely it is to be a product of random error. Titration curves, when performed, indicated that ratios of 0.5 and 1.5 times the mean value translate into approximately threefold decreases or increases in MAb affinity, respectively.

Certain gp120 mutants were also assessed for their ability to bind some of the C4 MAbs by the radioimmunoprecipitation assay (RIPA) procedures described previously (39, 41). The assays were done with secreted gp120, and no detergent was present. When we mapped the same MAb (e.g., human MAbs 48d and 15e) by the ELISA method and by RIPA, we found that the two methods yielded results that were in excellent agreement (40). Thus, it is unlikely that capture of gp120 onto a solid phase via its C terminus creates significant artifacts affecting its reactivity with most MAbs.

RESULTS

Binding of C4 MAbs to peptides and native and denatured gp120s. To evaluate the properties of MAbs against the C4 region, we used a panel of five mouse MAbs (G3-42, G3-299, G3-508, G3-519, and G3-536) raised against virus-derived IIIB gp120 (37), together with an additional murine MAb from the same source (G45-60) and a rat MAb (ICR 38) raised against recombinant BH10 gp120 (5). All the MAbs were reactive with a C4-derived peptide, KQIINMWQKVGKAMYAPPIS, from the H×B2 sequence, consistent with previous reports (5, 37). Titration of either the coating peptide or the MAb revealed, however, that MAbs G3-42 and G3-299 had a 10- to 50-fold-lower affinity than the other C4 MAbs for the 20-mer peptide (data not shown). Fine mapping of the peptide epitopes for the murine MAbs with a set of six decameric peptides (BRU sequence) overlapping by two amino acids (22) showed that the core epitope for all except G45-60 was EVGKAMYAPP (equivalent to amino acids 429 to 438 of the BRU sequence, but note the E/K change at position 429 in the H×B2 sequence). However, the affinities of G3-42 and G3-299 for this peptide were very low (data not shown). The most reactive peptide for G45-60 was GKAMYAPPIS, although flanking peptides were also strongly bound.

To confirm that the poor reactivities of MAbs G3-42 and G3-299 with C4 peptides were not artifacts of the solid-phase method, we carried out competition assays (22, 25) with the decamer peptides (5 μg/ml). Peptide EVGKAMYAPP was the most effective solution-phase inhibitor of the binding of MAbs G3-508, G3-519, G3-536, and ICR 38 to native BH10 gp120. Peptides EVGKAMYAPP, GKAMYAPPIS, and AMYAPPISGQ potently inhibited G45-60 binding to gp120, whereas peptide YAPPISGQIR was ineffective despite the binding of G45-60 to this peptide in solid-phase assays (data not shown). However, none of the decamer peptides at concentrations of up to 30 μg/ml could compete significantly with G3-42 and G3-299 binding to gp120, although the 20-mer peptide KQIINMWQKVGKAMYAPPIS was an effective competitor for these and all the other C4 MAbs. Thus, the poor binding of G3-42 and G3-299 to the decamer C4 peptides adsorbed on a solid phase is also found in solution-phase assays. In contrast, rat MAb ICR 38 was unreactive with solid-phase decamer peptides but bound strongly to EVGKYAMYAPP in solution-phase assays. This distinguishes ICR 38 from G3-42 and G3-299 and suggests that the failure of ICR 38 to react with decamer peptides adsorbed on a solid phase is an artifact of these assays (22, 25).

The above experiments demonstrate that each of the C4 MAbs can bind a relatively short peptide sequence from the C4 domain but imply that there is variation in the way that different MAbs react with their core epitopes in the C4 region. One explanation could be that amino acids outside the core sequence contribute to the binding energy of the antibody-gp120 reaction. To assess how sensitive the interactions of the different MAbs with their core epitopes are to protein conformation, we determined the relative affinities of the C4 MAbs for native and denatured BH10 gp120 (Fig. 1). There were clear differences between the C4 MAbs in their recognition of native and denatured BH10 gp120. Whereas G45-60 and ICR 38 bound equivalently (±2-fold) to both forms of BH10 gp120, binding of G3-42 and G3-299 was almost completely lost after gp120 denaturation (approximately 300- to 1,000-fold reduction in binding). The reductions in affinity of G3-508, G3-519, and G3-536 for denatured compared with native BH10 gp120 were 10-, 5-, and 15-fold, respectively (Fig. 1 and data not shown). Thus, there was a reasonable correlation between the relative affinities of the C4 MAbs for C4 peptides and their abilities to bind to denatured gp120 (data not shown). The binding of C4 MAbs to BH10 gp120 was also affected by nonionic detergent; G3-42 and G3-299 binding was increased 3- to 5-fold by Nonidet P-40 treatment of gp120, while G3-508, G3-519, and G3-536 binding was reduced 10-, 5-, and 5-fold, respectively. G45-60 binding was relatively insensitive to nonionic detergent (data not shown). Each of the C4 MAbs was able to bind to both SF-2 and MN gp120s in the ELISA (data not shown).

Binding of some C4 MAbs to gp120 is inhibited by V3 loop sequence changes. Different MAbs clearly recognize the same, or a very similar, core peptide sequence in different ways on the native BH10 gp120 protein. MAbs G3-42 and G3-299, which reacted relatively poorly with C4 peptides, were also the most sensitive to the loss of native gp120 conformation. Taken together, these observations suggested that the epitopes for MAbs G3-42 and G3-299 are conforma-

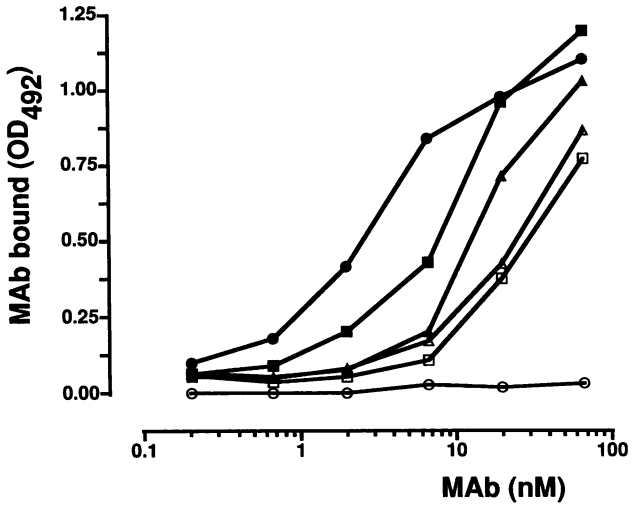

FIG. 1. Binding of C4 MAbs to native and denatured gp120. gp120 (BH10) was captured onto a solid phase via its carboxy terminus either as a native protein (solid symbols) or after denaturation by boiling in SDS-DTT (open symbols). MAbs at the concentrations indicated were then titrated, and bound MAbs were detected. MAbs: G3-299 (●, ○); G3-519 (■, □); G45-60 (▲, △). Data have been corrected for background absorption in the absence of gp120.

tion sensitive and could be discontinuous in nature. We therefore used a panel of gp120 mutants to assess whether their binding was influenced by alterations in the gp120 amino acid sequence outside their core epitope in C4. Each of the C4 MAbs was therefore tested in an ELISA against 63 gp120 mutants in an attempt to identify amino acid changes that completely abolished or seriously impaired binding (a reduction in binding of approximately threefold).

Not surprisingly, amino acid changes within the C4 core epitope had a significant effect on MAb binding (Table 1). None of the C4 MAbs bound detectably to the 433A/L and 435Y/H mutants, indicating that Ala-433 and Tyr-435 are critical components of the epitopes for each MAb. The 430V/S substitution abolished or very strongly impaired the binding of G3-299, G3-508, G3-519, and G3-536 and significantly reduced the binding of G3-42. This substitution, however, had no effect on the binding of G45-60, consistent with the peptide-binding data described above that indicated that the epitope for G45-60 was more carboxy terminal than those of the other MAbs. Only G3-519 and G3-536 were strongly affected by the 438P/R substitution, although it had weak effects on G3-42 and G3-508 binding. A double substitution (437-438PP/VV) was studied in a separate experiment and found to strongly inhibit the binding of all the MAbs except G3-42, on which it had little effect (Fig. 2 and data not shown). It was notable that the 432K/A change did not significantly reduce the binding of any C4 MAb, although Lys-432 is within the core peptide epitope EVGKAMYAPP for these MAbs. ICR 38 was analyzed in a separate experiment, in which its binding was found to be strongly sensitive to the 430V/S, 433A/L, 435Y/H, 437-438PP/VV, and 438P/R amino acid changes and to be weakly inhibited by the 427W/S substitution (data not shown).

A few amino acid substitutions outside the C4 region (36V/L, 450T/N, and 463N/D) were weakly inhibitory for

TABLE 1. Effect of amino acid sequence changes on the binding of C4 MAbs to H×B2 gp120[a]

gp120 mutation	OD_{492} ratio					
	G3-42	G3-299	G3-508	G3-519	G3-536	G45-60
Inhibitory						
36V/L	0.87	0.80	**0.41**	**0.41**	**0.46**	**0.63**
308-310RIQ/RPELIPVQ	**0.00**	**0.00**	1.05	1.37	0.67	1.65
314G/W	**0.24**	**0.09**	**0.33**	**0.33**	**0.12**	**0.60**
430V/S	**0.30**	**0.08**	**0.12**	**0.14**	**0.00**	**1.04**
433A/L	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.02**
435Y/H	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
438P/R	**0.48**	0.76	**0.43**	**0.18**	**0.24**	1.22
450T/N	**0.39**	0.98	0.59	**0.44**	**0.41**	0.92
463N/D	1.17	0.89	0.73	**0.28**	**0.46**	1.05
Enhancing						
69W/L	**1.81**	1.71	**1.41**	**1.71**	1.43	1.71
117K/W	1.64	1.44	**1.62**	**1.65**	**2.05**	**2.14**
257T/G	1.45	1.54	1.43	**1.64**	1.20	1.94
257T/R	1.77	**1.82**	1.48	1.39	**1.89**	**2.03**
262N/T	**2.32**	**2.54**	**2.29**	**3.60**	**2.05**	**3.65**
298R/G	1.68	1.44	1.04	**1.90**	**1.63**	**2.02**
370E/Q	1.58	**1.84**	1.32	**1.62**	**1.89**	**2.09**
370E/R	1.77	1.72	1.40	1.40	**1.57**	**1.95**
475M/S	**2.11**	**2.20**	**1.56**	1.34	1.45	**2.52**
Mean ratio ± SD[b]	1.19 ± 0.38	1.21 ± 0.40	0.94 ± 0.33	0.99 ± 0.36	0.98 ± 0.40	1.30 ± 0.43

[a] Data for each of the MAbs were derived from triplicate determinations except for G3-299, which was analyzed in quadruplicate. MAbs were used at 3.5 nM except for G3-508 (10 nM) and G45-60 (7 nM). Values indicating binding ratios of <0.5 times (inhibitory) or >1.5 times (enhancing) the mean value for the panel are listed in boldface type. The following mutants plus wild-type H×B2 gp120 comprised the test panel: 36V/L, 40Y/D, 45W/S, 69W/L, 80N/R, 88N/P, 102E/L, 103Q/F, 106E/A, 113D/A, 113D/R, 117K/W, 120/121VK/LE, 125L/G, Δ119-205, 207K/W, 252R/W, 257T/R, 257T/G, 262N/T, 266A/E, 267E/L, 269E/L, 281A/V, 298R/G, 308-310RIQ/RPELIPVQ, 314G/W, 356N/I, 368D/R, 368D/T, 370E/R, 370E/Q, 380G/F, 381E/P, 382F/L, 384Y/E, 386N/Q, 392N/E plus 397N/E, 395W/S, 406N/G, 420I/R, 421K/L, 427W/V, 427W/S, 429K/L, 430V/S, 432K/A, 433A/L, 435Y/H, 438P/R, 450T/N, 456R/K, 457D/A, 457D/R, 463N/D, 470P/L, 475M/S, 485K/V, 491I/F, and 493P/K. Other V3 mutants were tested by RIPA (see text).

[b] Average value of the OD_{492} ratio of the test MAb to the whole panel of reference sera (see text).

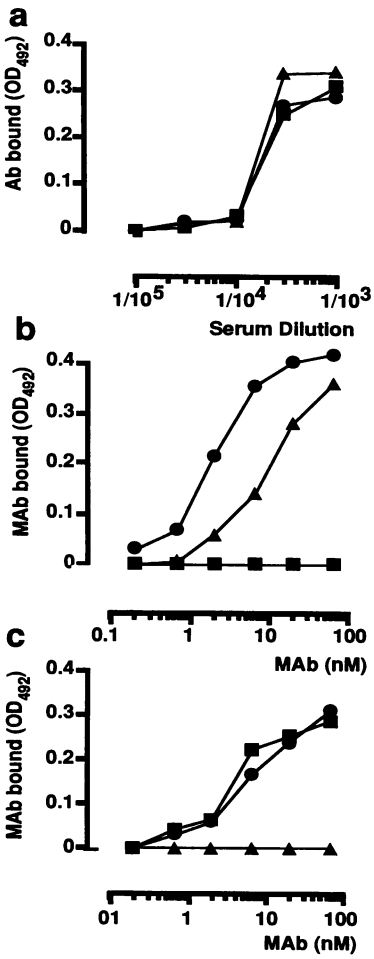

FIG. 2. Binding of antibodies to wild-type gp120 and a V3 loop mutant. (a) Equivalent amounts of wild-type H×B2 gp120 (●), a V3 loop insertion mutant (308-310RIQ/RPELIPVQ) (■), or a C4 mutant (437-438PP/VV) (▲), as judged by the binding of HIV-1$^+$ human serum, were captured onto the solid phase via a carboxy-terminus antibody. C4 MAbs G3-42 (b) and G3-508 (c) were titrated against the captured gp120s, and the amount of bound MAb was determined.

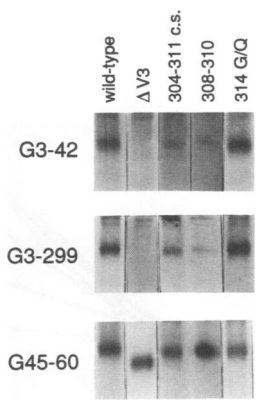

FIG. 3. Effect of V3 amino acid substitutions on gp120 binding of C4 MAbs. Precipitation from transfected COS-1 supernatants of wild-type or mutant HIV-1 (H×B2) gp120 glycoproteins by MAbs G3-42, G3-299, and G45-60. Precipitations and washes were performed in the absence of detergent.

some of the C4 MAbs (Table 1), but their significance remains to be evaluated by independent techniques. However, one amino acid change outside C4 completely destroyed the ability of gp120 to bind some C4 MAbs. A five-amino-acid insertion in the V3 loop (308-310RIQ/RPE LIPVQ) abolished binding of G3-42 and G3-299 to gp120 but had no significant effect on binding of G3-508, G3-519, G3-536, G45-60, or ICR 38. This was confirmed when titration curves were prepared (Fig. 2). It is clear that G3-42 fails to bind to the V3 loop mutant (Fig. 2b) but that G3-508 (Fig. 2c) binds approximately as well to the mutant as to the wild-type glycoprotein. Similar titration curves were prepared for other C4 MAbs; G3-299, like G3-42, totally failed to bind the V3 loop mutant, while there was an approximately twofold reduction in the affinities of G3-519 and G3-536 for the mutant compared with affinities for wild-type gp120 (data not shown). However, HIV-1$^+$ serum and a control MAb, B15, to the V4 domain bound equivalently to the V3 mutant and to wild-type gp120 (Fig. 2a and data not shown), as do several MAbs to the V2 domain (29) and to regions around the CD4 binding site (23, 39, 41). It should be noted that most anti-gp120 antibodies in the HIV-1$^+$ serum sample recognize conformation-sensitive epitopes (26). Thus, the V3 loop insertion does not have global effects on gp120 structure; indeed, it does not significantly affect the binding of MAbs to any domain of gp120 other than V3 or C4 (see Table 3; data not shown). A point substitution in V3, 314G/W, also reduced the binding of each of the C4 MAbs to gp120 (Table 1). Binding of G3-299 and G3-536 was virtually abolished by the 314G/W change, but its effect on the binding of G3-42, G3-508, and G3-519 was weak and on that of G45-60 was very weak.

The influence of V3 sequence changes on the binding of G3-42, G3-299, and G45-60 to gp120 was also examined by RIPA procedures in the absence of detergent (Fig. 3), using secreted H×B2 gp120 (39, 41). None of the changes listed below affected the binding of G45-60, consistent with the ELISA data suggesting that V3 sequences are unimportant for its binding. However, complete deletion of the V3 loop abolished the ability of gp120 to bind G3-42 and G3-299, and there was barely detectable binding of these MAbs to the 308-310RIQ/RPELIPVQ mutant (Fig. 3). The 314G/W substitution significantly reduced the binding of G3-299 but had little effect on G3-42 binding. The other mutant (304-311c.s.) showing strong reduction in binding of both G3-42 and G3-299 had a complex set of changes in the amino-terminal side of the V3 loop (304R/K plus 305K/R plus 306R/K plus 308R/K plus 311R/K). The following V3 changes had no effect on the binding of either G3-42 or G3-299: 308R/H, 313P/S, 313P/L plus 315R/Q, 314G/Q, and 315R/G (Fig. 3 and data not shown).

There was an increase in the binding of C4 MAbs to a few gp120 mutants in the ELISA experiments (Table 1). A sequence change at the base of the V3 loop (298R/G) was enhancing for several of the C4 MAbs, consistent with a prior report that this substitution increases the binding of MAbs to epitopes that overlap the CD4-binding site and also to the V3 loop (45) (see Table 3). A glycosylation site mutant (262N/T) had a large enhancing effect for the C4 MAbs, and amino acid changes at the nearby Thr-257 residue were also weakly enhancing. It must be noted, however, that the 262N/T mutant was poorly secreted and so was present in the culture supernatants only at low concentrations. Thus,

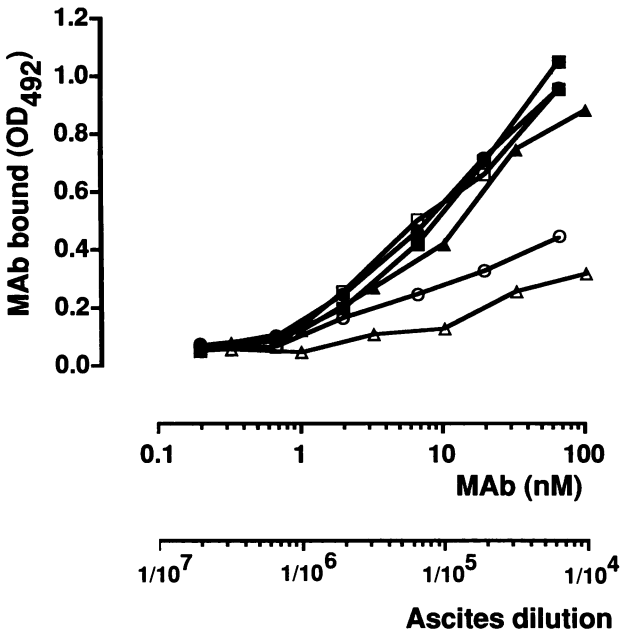

FIG. 4. Effect of thrombin cleavage of the gp120 V3 region on the binding of C4 and V3 MAbs to gp120. The binding of MAbs G3-299 (○, ●), G45-60 (□, ■), and 110.5 (△, ▲) to BH10 gp120 with (open symbols) or without (solid symbols) prior treatment with thrombin is indicated. MAb 110.5 was used in the form of ascites fluid; the other two were used as purified antibodies.

apparent increases in binding are based on small changes in ELISA OD_{492} values. The same caveat applies to the 475M/S mutant, which was also enhancing for some of the MAbs. Substitutions at Glu-370 apparently increased the binding of several C4 MAbs to gp120. However, we often find this to be the case for MAbs that do not include this amino acid in their probable epitope. This might be due to a reduction in the ability of HIV-1^+ serum to bind to this gp120 mutant, consistent with results described elsewhere (23, 26), thereby giving an apparent enhancement in MAb binding because of our practice of determining the ratio of MAb/HIV-1^+ serum binding for each mutant. Thus, although it is intriguing that sequence changes around amino acids Thr-257, Met-475, Glu-370, and Asp-113 (Lys-117), which are all implicated in the binding of CD4 (32), or anti-CD4-binding-site MAbs (39, 41), can apparently influence the presentation of the C4 domain, these observations will require confirmation by independent techniques.

Two C4 MAbs are sensitive to V3 loop proteolysis, and one binds a V3 peptide. To further explore the influence of the V3 loop on the binding of C4 MAbs to gp120, we treated BH10 gp120 with thrombin to cleave the V3 loop between Arg-315 and Ala-316. Previous studies have shown that thrombin cuts uniquely at this site under these conditions (4, 33). This scission reduced binding of G3-299 by an extent comparable with the loss of binding of the V3 MAb 110.5 (Fig. 4). The epitope for the latter MAb is known to be destroyed by thrombin cleavage (4, 33); residual binding of 110.5 and G3-299 to thrombin-treated gp120 probably represents the presence of uncleaved gp120 molecules. G3-42 binding was also reduced by thrombin treatment but not to quite the same extent as G3-299 binding (data not shown). In contrast, the binding of G45-60 was unaffected (Fig. 4), indicating that the loss of G3-299 binding was not due to an unexpected cleavage of gp120 in the C4 region.

G3-42 and G3-299, both sensitive to V3 loop sequence changes (Table 1), are the two C4 MAbs least able to bind denatured gp120 or C4 peptides (Fig. 1). These observations suggest that C4 amino acids might constitute only part of the epitope for these MAbs. To investigate this, we determined whether these MAbs could bind to peptides from the V3 region of gp120 (Table 2). G3-299 was able to bind weakly to solid-phase-adsorbed peptides derived from the V3 region of H×B2 or BRU gp120, but no other C4 MAb, including G3-42, could bind these peptides. G3-299 failed to bind to a peptide from the V3 region of SF-2 gp120, suggesting that its interactions with the H×B2 and BRU peptides were sequence specific. Binding of G3-299 to the 36-mer peptide incorporating the entire BRU V3 region was greater than to a shorter peptide encompassing the amino-terminal side and the central part of the H×B2 V3 region (CNTRKRIRI QRGPGRAFVTIGK) (Table 2), and two shorter peptides from the H×B2 sequence (NNTRKRIRIQRGPGRAFVTI and RGPGRAFVTIGKIGNMRQAH) were not bound significantly by G3-299 (data not shown). This might be explained by the greater conformational flexibility of longer peptides and/or by the requirement for sequences in both flanks of the V3 region for G3-299 binding. Titration of G3-299 against the cyclic BRU 36-mer V3 peptide and against the 20-mer C4 peptide (see above) coated at 10 μg/ml indicated that the affinity of G3-299 for the V3 peptide was approximately 150-fold lower than for the C4 peptide.

V3 MAbs can inhibit the binding of C4 MAbs to gp120. The above data suggested that the V3 region of gp120 had

TABLE 2. Binding of G3-299 to V3 peptides[a]

Peptide	OD_{492} in ELISA with MAb:							
	G3-42	G3-299	G3-508	G3-519	G3-536	G45-60	ICR 38	9284
H×B2 V3	0.018	**0.206**	0.036	0.027	0.049	0.029	0.044	**1.560**
SF-2 V3	0.019	0.018	0.040	0.019	0.040	0.059	0.037	**1.017**
BRU V3 cyclic	0.020	**0.323**	0.055	0.030	0.048	0.058	0.037	**1.455**
BRU V3 linear	0.020	**0.262**	0.049	0.021	0.047	0.030	0.040	**1.426**
H×B2 421–440	**1.377**	**1.167**	**1.500**	**1.496**	**1.445**	**1.595**	**1.352**	0.036
H×B2 735–752	0.025	0.019	0.023	0.028	0.026	0.021	0.053	0.037

[a] The MAbs indicated were tested at 2 μg/ml for their ability to react with various peptides adsorbed to a solid phase at 10 μg/ml. MAb 9284 is a control MAb to the V3 region of HIV-1 IIIB. OD_{492} values of >0.100 are shown in boldface type. The peptides used were H×B2 V3 from American Biotechnologies (CNTRKRIRIQRGPGRAFVTIGK); SF-2 V3 from American Biotechnologies (CNTRKSIYIGPGRAFHTTGR); BRU V3, cyclized and linear, from the ADP Reagent Repository (CTRPNNNTRRSIRIQRGPGRAFVTIGKIGNMRQAHCN); C4 amino acids 421 to 440 from Cambridge Research Biochemicals (KQIINMWQKVGKAMYAPPIS); and gp41 amino acids 735 to 752 from the ADP Reagent Repository (DRPEGIEEEGGERDRDRS).

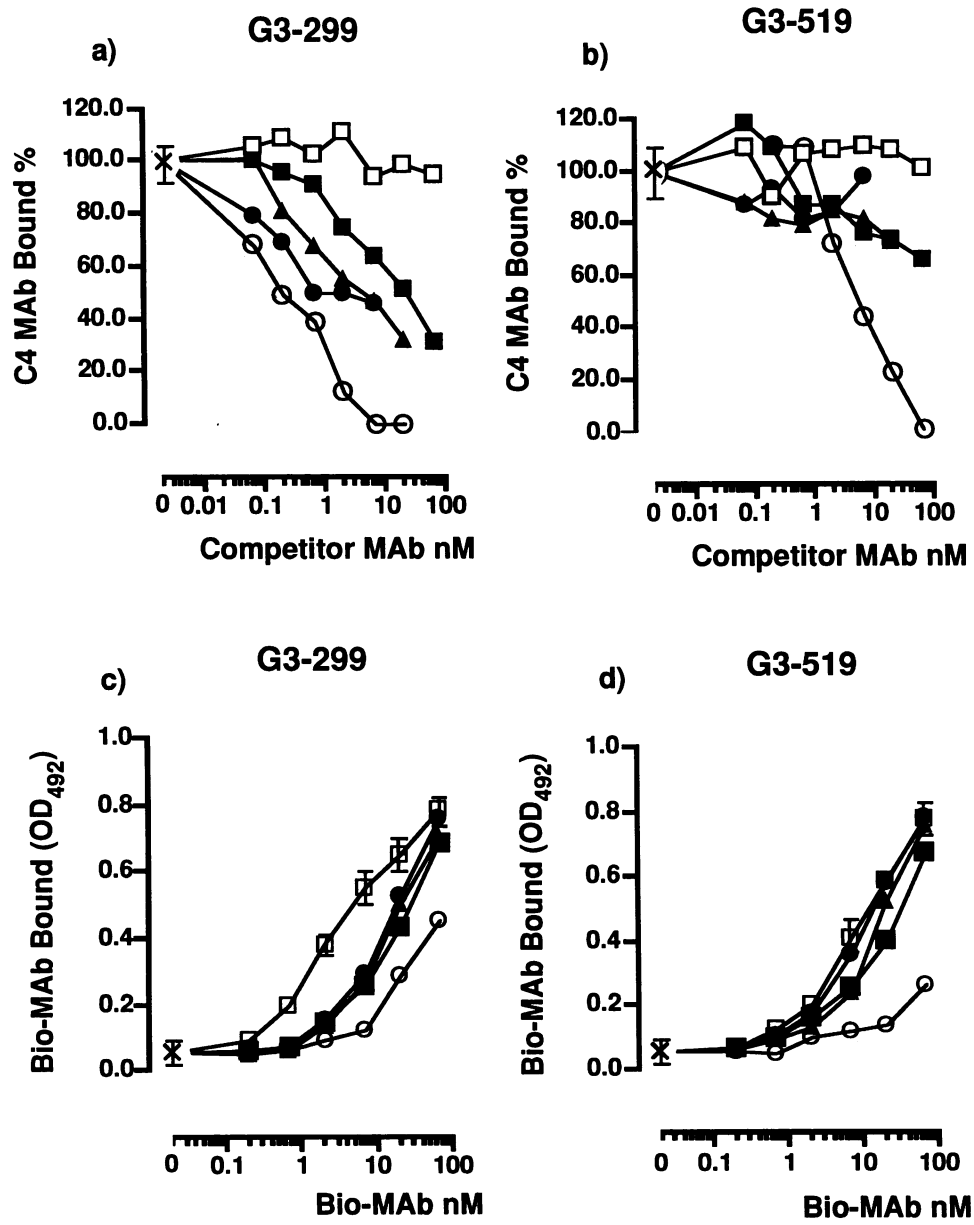

FIG. 5. Cross-competition with C4 MAb binding by V3 loop MAbs. (a and b) Native BH10 gp120, immobilized on D7324, was reacted with competitor MAbs at the concentrations indicated before addition of (a) biotin-G3-299 (6.7 nM) or (b) biotin-G3-519 (20 nM) and determination of the amount of bound biotin-labeled MAb. The competitor MAbs were (▲) 9284 (amino side of V3); (●) 110.5 (crown of V3); (■) 110.I (carboxy side of V3); (○) G3-508 (C4); and (□) 684-238 (V2). For 110.5, we assumed that the ascites fluid contained 30 mg of MAb per ml. The 100% binding value (no competitor) corresponded to OD_{492} values of 0.843 and 0.748 and 0% binding (no gp120) corresponded to OD_{492} values of 0.080 and 0.087 in panels a and b, respectively. (c and d) The following competitor MAbs were added at saturating concentrations to gp120 before titration of (c) biotin-G3-299 or (d) biotin-G3-519 and determination of the amount of biotin-labeled MAb. Additions: (□) no competitor; (▲) 20 nM 9284; (●) 67 nM 110.5; (■) 20 nM 110.I; (○) 67 nM G3-508. Data points are the means of duplicate determinations except for the no-competitor control, for which they are the means ± standard deviation of triplicates. The omission of an error bar means that it lies within the symbol.

considerable influence on the binding of some MAbs to the C4 region. We explored this further by cross-competition experiments, in which the binding of biotin-labeled C4 MAbs was assessed in the presence of other, unlabeled MAbs (Fig. 5a and b). First, the competitor MAbs were added in increasing amounts up to the concentrations previously determined to give saturation binding. As controls, the binding of both G3-299 and G3-519 was shown to be completely blocked by the C4 MAb G3-508, whereas MAb 684-238 to the V2 loop had no inhibitory effect (Fig. 5a and b). It was notable that higher concentrations of G3-508 were required to inhibit G3-519 binding than to inhibit G3-299 binding. As G3-519 has a lower affinity than G3-299 for gp120 (Fig. 1), this quantitative difference in G3-508 blocking

TABLE 3. Effect of amino acid sequence changes on binding of V3 MAbs to H×B2 gp120[a]

gp120 mutation	OD_{492} ratio					
	110.5	0.5β	5021	5042	F58/D1	P1/D12
Inhibitory						
314G/W	0.00	0.00	0.00	0.00	0.00	0.00
281A/V					0.34	
308-310RIQ/RPELIPVQ		0.00		0.00	0.38	
450T/N			0.33	0.07		
406N/G				0.14		
Enhancing						
262N/T	1.68					
430V/S		1.25				
308-310RIQ/RPELIPVQ			1.43			
298R/G			1.08	0.67		
427W/S					1.81	
384Y/E						1.63
432K/A		1.23		0.57		
252R/W				0.59		
420I/R			0.97	0.59	1.73	
475M/S					1.62	
429K/L					1.35	
Mean ratio ± SD[b]	0.96 ± 0.25	0.80 ± 0.21	0.66 ± 0.20	0.38 ± 0.13	0.90 ± 0.29	1.08 ± 0.28

[a] Data for each of the MAbs were derived from triplicate determinations except for 5021 and P1/D12, which were analyzed in duplicate. The MAb concentrations used were: 110.5, 1:20,000 dilution of ascites; 0.5β, 1:3,000 dilution of ascites; 5021, 1 μg/ml; 5042, 5 μg/ml; F58, 1 μg/ml; and P1/D12, 0.3 μg/ml. Only values indicating binding ratios of <0.5 times (inhibitory) or >1.5 times (enhancing) the mean value are listed. The mutant panel was the same as listed in Table 1, footnote *a*, with the addition of 438PP/VV and 477D/V. However, the stock of transfection supernatants was different.

[b] See Table 1, footnote *b*.

efficiency may reflect the greater ease with which the conformation-sensitive G3-299 epitope is disrupted by a competitor.

MAb G3-299 binding was also inhibited by each of three V3 loop MAbs: 9284 to the amino-terminal side; 110.5 to the crown, and 110.I to the carboxy-terminal side (Fig. 5a), although complete inhibition was not achieved. In contrast, binding of the V3 sequence-insensitive MAb G3-519 was not inhibited by 110.5 and was only very weakly inhibited by 9284 and 110.I (Fig. 5b). Although very weak, the inhibitory effect of MAbs to the V3 flanks on G3-519 binding was consistent between experiments. This was confirmed when G3-299 and G3-519 were titrated in the presence of saturating concentrations of competitor MAbs (Fig. 5c and d); MAbs 110.I and 9284 but not 110.5 weakly inhibited G3-519 binding, whereas all three V3 MAbs inhibited G3-299 binding (Fig. 5c). In other experiments, the binding of G3-42, G3-536, and G45-60 was also inhibited by the above V3 MAbs, G3-42 being especially sensitive (data not shown). Control MAbs to the V2 loop of gp120 had no effect on the binding of any of the C4 MAbs (data not shown). Thus, the antibody inhibition data support the conclusion that there is a close structural interaction between the V3 and C4 regions of gp120.

Effect of gp120 amino acid substitutions on the binding of V3 loop MAbs. Wyatt et al. have shown that the binding of a MAb to the amino-terminal flank of the V3 loop is enhanced by sequence changes at the base of the V3 loop and in the C4 domain, as is the binding of MAbs such as 15e to epitopes that overlap the CD4 binding site (45). We sought to establish how general this phenomenon was for V3 loop MAbs by using MAbs that recognized the more-potent neutralization epitope at the crown of this loop. Each of the MAbs tested is capable of recognizing a V3 loop peptide, and the core epitopes are as follows: 110.5, IQRGPGRAF (16); 0.5β, GPGRAFVTIG (34); 5021, QRGPGRA (8, 9); 5042, QRGPGRA (8, 9); F58/D1, I..GPGRA (2, 3); P1/D12, I..GPGRA (2); and P4/D10, I..GPGRA (2, 3). Despite the similarity in the peptide epitopes for these MAbs, there was considerable variation in their relative abilities to recognize native and denatured BH10 gp120. In an experiment similar in design to that of Fig. 1, it was found that the approximate losses of affinity for gp120 upon denaturation were: 110.5, 100- to 300-fold; 0.5β, 100- to 300-fold; 5021, 100- to 300-fold; 5042, 300-fold; F58/D1, 1- to 3-fold; P1/D12, 1- to 3-fold; P4/D10, 3-fold. Thus, as found for the C4 MAbs, V3 MAbs can be sensitive to the way in which their epitopes are presented on the native protein, but this cannot be predicted from their ability to bind peptides (25). Nor does sensitivity to protein conformation correlate with the immunogens against which the MAbs were raised: 5021 and 5042 are antipeptide MAbs; the immunogen for the others was native, virus-derived gp120. However, the shortness of the peptide epitopes for MAbs F58/D1, P1/D12, and P4/D10 (2, 3) might account for their relative insensitivity to protein conformation.

Binding of all of the V3 loop MAbs was abolished by the 314G/W substitution within their peptide epitope (Table 3). However, the insertion of amino acids into the V3 loop (308-310RIQ/RPELIPVQ) had no effect on the binding of two of the V3 MAbs, had only a weak effect on a third, and actually enhanced the binding of MAb 5021. Thus, although a fairly drastic change, the insertion of five amino acids does not destroy the structure of the V3 loop, further arguing that its effect on C4 MAb binding (Fig. 2, Table 1) is likely to be specific. Sequence changes outside of V3 tended not to be inhibitory for V3 MAb binding, although MAb 5042 was weakly sensitive to two substitutions that removed glycosylation sites in C4 and V4 (406N/G and 450T/N) (Table 3). Complete removal of the V1 and V2 loops was generally moderately inhibitory for V3 loop MAb binding (data not shown). The significance of these and other mildly inhibitory changes is under investigation.

Several substitutions enhanced the binding of different MAbs (Table 3), although in some cases (370E/R, 262N/T, and 475M/S), the caveats noted above (Table 1) apply. A change in C3 at amino acid 370 (370E/R) was apparently enhancing for each MAb (data not shown). Removal of a glycosylation site in C2 (262N/T) weakly enhanced the binding of 110.5 but of no other MAb in two of three experiments. An association between this glycosylation site and the V3 loop has been suggested previously (44). Whether the weak effect of the 262N/T change on 110.5 binding has any biological relevance must await further studies. Another substitution in C2 (252R/W) moderately increased binding of MAb 5042 (Table 3). An amino acid change at the amino-terminal base of the V3 loop (298R/G) enhanced the binding of two V3 MAbs, consistent with the prior report of the effect of this substitution on the binding of MAb 9284 to the V3 loop (45). Except for a single amino acid change in C5 (475M/S) that enhanced the binding of MAb F58/D1, all of the other enhancing substitutions were in C4 or in C3, at the base of the V4 loop. We commonly find that amino acid changes between positions 380 and 385 in C3 are similar to substitutions between positions 420 and 432 in C4 at the other end of the V4 loop in their effects on the binding of V3 and other MAbs (data not shown). These two regions of gp120 may be proximal in the folded protein. In addition to the mutants listed in Table 3, it is notable that other mutants with changes in the above C3 and C4 regions were generally among the gp120s giving the six highest binding ratios for each MAb, even if the values were below our cutoff level for significance (data not shown). Thus, the data support the conclusions of Wyatt et al. (45) and those we drew from the data in Fig. 2: there appears to be an interaction between the V3 and C4 domains of gp120.

Assessing the topology of the C3/C4/V4 region by immunochemistry. To gain initial insights into the possible structure of gp120 domains, we probed native and denatured gp120 with antibodies to the C4 and V4 loops and the adjacent segment of C3 to see which regions of the molecule were accessible to antibody binding. It is clear from Fig. 1 that the EVGKAMYAPP sequence (positions 429 to 438) in C4 is exposed on the surface of the native, recombinant protein. However, a rabbit antiserum to the peptide sequence positions 436 to 450 flanking the EVGKAMYAPP segment bound only to denatured gp120 and did not recognize the native protein (Fig. 6a). Another antiserum to amino acids 416 to 431 also bound only to denatured gp120, but the level of binding was too weak for any conclusions to be drawn. The EVGKAMYAPP sequence is probably part of a surface loop protruding from the interior of gp120. Precisely how much of the regions flanking this sequence are exposed for antibody binding is not known. Additional MAbs to this domain would assist in the definition of its structure.

We used four MAbs whose epitopes map between amino acids 378 and 390 to probe the exposure of the conserved region of the C3 domain at the amino-terminal base of the V4 loop (Fig. 6b). The epitopes for these MAbs were identified initially by using the gp120 mutant panel as described above, except that transfected-cell lysates were used after boiling in SDS-DTT to denature the gp120 and gp160 molecules present (26). Changes between amino acids 380 and 390 generally abolished binding of these MAbs to denatured gp120. For example, MAb C12 did not bind the 380G/F, 381E/P, or 382Y/E mutants, and the 356N/I, 370E/R, 386N/R, and 392N/E plus 397N/E substitutions caused a moderate reduction in binding (data not shown).

To further define the epitopes, we used peptides. MAb

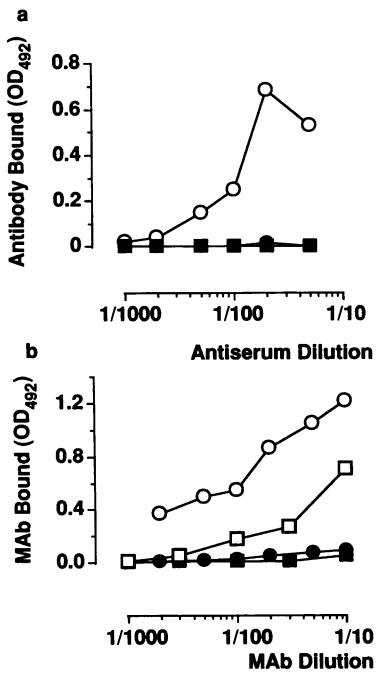

FIG. 6. Probing the topology of the V4 and C4 loops with MAbs. Native (solid symbols) and denatured (open symbols) BH10 gp120 was reacted with (a) rabbit antiserum R213 (●, ○) to amino acids 436 to 450 or (b) MAbs B32 (●, ○) to amino acids 380 to 393 and C12 (■, □) to amino acids 370 to 380. Data have been corrected for background absorption of antibodies to the solid phase at each antibody concentration in the absence of gp120.

C12 bound peptide 362–381 (OD_{492}, 1.315) but not peptide 350–370 or 380–393. This localizes its epitope to approximately amino acids 371 to 381, although amino acids outside this sequence can also influence its binding. MAb C12 also bound strongly to denatured MN gp120. There is only a single amino acid change between BH10 and MN gp120 in the 371 to 381 region, 373T/M. MAb 36.1 bound to peptide 362–381 (OD_{492}, 1.864) but did not recognize peptide 350–370 or 380–393; its epitope is probably very similar to that of C12. MAb B32 recognized peptide 380–393 (OD_{492}, 0.949) but not peptide 362–381 or 392–411, suggesting that its epitope is within amino acids 380 to 392. B32 also bound strongly to denatured MN gp120, so its epitope may well be within the sequence GEFFYCN (380 to 386), which is completely conserved between MN and BH10 gp120s. The only peptide bound by MAb 110.D was 380–393 (OD_{492}, 0.221), implying that its epitope was similar to that of B32.

When we used recombinant BH10 gp120 to test the reactivity of the above C3 MAbs with a native form of the protein, we found that no MAb bound significantly (Fig. 6b). However, each MAb bound strongly to denatured, recombinant gp120, exemplified by C12 and B32 (Fig. 6b). Thus, the region of gp120 between amino acids 370 and 393 is likely buried in the interior of the native molecule and not exposed on its surface for MAb binding. Note that the inclusion of SDS is not necessary for exposure of these buried epitopes, as boiling in the presence of DTT suffices. However, nonionic detergents alone are not capable of exposing the buried epitopes (data not shown).

The V4 MAb B15 bound reasonably well to native BH10 gp120; half-maximal binding occurred at approximately 5 to

10 μg/ml (data not shown). This MAb also bound strongly to peptide 392–411 (OD_{492}, 0.928) but not to the flanking peptides 380–393 and 402–420. Thus, its core epitope is within amino acids 392 to 402 (1). B15 failed to bind to either native or denatured MN gp120 (data not shown), which is consistent with the extensive sequence variation in the V4 sequences of MN and IIIB gp120s. When tested against the H×B2 mutant panel under native conditions without detergent, MAb B15 completely failed to bind to the 395W/S mutant, and its binding to the 386N/R mutant was significantly reduced. These amino acid changes are within or close to the peptide epitope defined above. Trp-395 is conserved in most published HIV-1 gp120 sequences but is replaced in the Chiang Mai northern Thailand isolate gp120 by the amino-terminal one of a pair of cysteines that presumably form an extra disulfide bond near the crown of the V4 loop (20). A substitution in C4 (427W/S) also weakly enhanced B15 binding to gp120 (data not shown). Thus, the presentation of the V4 domain may be influenced by changes in C4, although this requires further evaluation.

It is unlikely that the B15 epitope is discontinuous. First, it was raised against MicroGeneSys gp160, which is a denatured protein (1). Second, denaturation of our BH10 gp120 increased its affinity for MAb B15 by about fivefold, indicating that the B15 epitope is linear but not optimally exposed on the surface of native gp120 (data not shown). Either part of the epitope is occluded by other regions of gp120, or else access of the MAb to its epitope is hindered by the extensive glycosylation of the V4 loop. Nonionic detergent treatment of gp120 actually reduced B15 binding by approximately fivefold (data not shown).

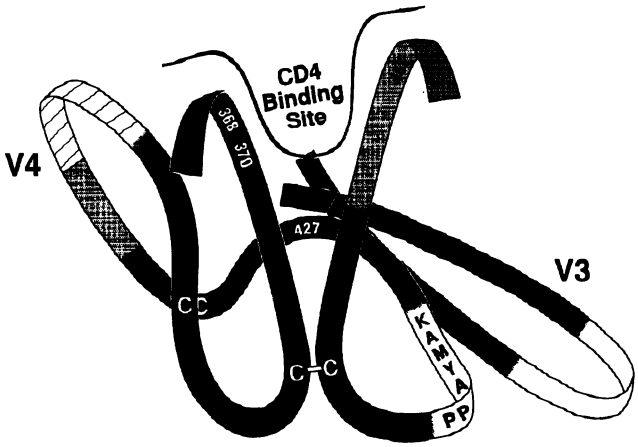

FIG. 7. Model for the HIV-1 gp120 V3-C3-V4-C4 regions. A low-resolution schematic model for the HIV-1 gp120 regions extending from residue 365 in C3 to residue 460 in C4 is shown. The linear regions shown to be buried in the native gp120 glycoprotein are black, while the linear regions accessible to antibodies are white. Regions for which no data are currently available are stippled. The disulfide linkages between residues 378 and 445 and between residues 384 and 418 are shown (C—C). The approximate location of the CD4 binding site is indicated, with gp120 amino acids important for CD4 binding (Asp-368, Glu-370, Trp-427, and Asp-457) highlighted. The relationship between the amino-terminal side of the V3 loop and the C4 region is also shown. Data allowing orientation of the V4 loop with respect to the other structures are not available, so its position on the diagram was chosen arbitrarily. The hatched V4 region represents the epitope for the B15 MAb, the binding of which is moderately increased (fivefold) by gp120 denaturation.

DISCUSSION

The conservation of the amino acid sequence of the C4 region of HIV-1 gp120 among divergent isolates implies a crucial role for this domain. Trp-427, located in the C4 region, has been identified as important for binding of gp120 to CD4 (6, 32). Most other amino acid substitutions in C4, including those affecting the highly conserved EVGKAMY APP sequence (residues 429 to 438), do not affect CD4 binding (32). These results are consistent with a model in which the CD4 binding site of gp120 is composed of amino acids derived from discontinuous regions of the glycoprotein, including C3, C4, and possibly C2. The results also imply that functional roles other than CD4 binding probably account for the observed sequence conservation of the C4 region. Relevant to this, it has been shown that amino acid changes in C4 can affect post-receptor binding functions involved in virus entry (38) and also modify the affinity of the association between the gp120 and gp41 glycoproteins (11).

The C4 domain is immunogenic in HIV-infected humans, demonstrated by the reactivity of HIV-1^+ sera with modified C4 peptides (43). Is it a domain relevant to virus neutralization in the natural humoral immune response to HIV-1 infection? The EVGKAMYAPP segment of C4 is well exposed on the surface of monomeric gp120 (Fig. 1), yet the C4 MAbs against this region are generally poorly neutralizing in comparison to MAbs that bind to discontinuous epitopes overlapping the CD4 binding site, and especially in comparison to V3 MAbs (5, 7, 17, 31, 37, 39, 41) (data not shown). It is possible that MAbs to other regions of C4 may be more potent neutralizing antibodies than those we have yet examined. However, the C4 MAbs in our test panel bind very poorly to oligomeric gp120 on the surface of IIIB-infected cells (33a); this may account for their relative weakness as neutralizing antibodies.

Two of the C4 MAbs, G3-42 and G3-299, are clearly distinguishable from the other five by their failure to react with denatured gp120; their relatively low affinity for C4-derived peptides, especially short ones; their sensitivity to amino acid changes in the V3 region and to thrombin cleavage of the V3 loop; and their susceptibility to blockage by V3 loop MAbs. The available data do not allow us to distinguish whether these antibodies recognize a linear C4 epitope influenced in its conformation by V3 structure or a discontinuous epitope composed of both C4 and V3 sequences. However, the binding of V3 peptides by the G3-299 MAb might indicate that V3 sequences contribute some of the binding energy to the MAb-gp120 interaction. In either case, the results extend our ability to model the relationship between the V3 loop and the C4 region suggested by Wyatt et al. (45). In that study, a relationship between the base and amino-terminal side of the V3 loop and the CD4 binding region, including Trp-427, was inferred. The present study suggests that a close relationship exists between the V3 loop and C4 structures within or near the EVGKAMYAPP sequence. That MAbs whose epitopes map to the sides of the V3 loop are more potent than one whose epitope maps to its crown at blocking G3-519 binding to gp120 (Fig. 5b and data not shown) suggests that it might be the V3 flanks, rather than the crown, that are closest to C4 structures, but this is a tentative conclusion. As G3-42 and G3-299 also bind strongly to MN and SF-2 gp120s (data not shown), the V3/C4 relationship is not likely to be restricted to gp120 from the IIIB isolate. Furthermore, independent evidence in support of an interaction between the V3 and C4 regions of gp120s

from primate lentiviruses has been provided by studies on a simian immunodeficiency virus strain (SIV_{mac}) (30). In this virus, a Val-Gly substitution at gp120 amino acid 448 (equivalent to Met-434 of H×B2) that reduces viral infectivity was compensated for by a Val-Ile change at amino acid 322 (equivalent to Ile-307 of H×B2) (30). These SIV amino acids are remarkably similar in position to those implicated in the recognition of MAbs G3-299 and G3-42 on H×B2 gp120 (Table 1). In addition, a strong neutralization epitope of SIV_{mac} appears to be a composite structure, incorporating elements of the V3, V4, and C4 domains (12). Thus, there may be significant similarities in the structure-function relationships of the envelope glycoproteins of these different but related lentiviruses (28, 30).

The simplest explanation for these observed relationships is a structural proximity of the V3 loop and C4 sequences, with the V3 loop oriented with its base near the CD4 binding site and the amino-terminal side extending alongside the C4 region from residues 429 to 438. A schematic representation of this is shown in Fig. 7. This structural model helps explain the observation that soluble CD4 binding can increase the exposure of the HIV-1 gp120 V3 loop (4, 33). The bases of the V3 loop are known to be poorly exposed on native gp120, consistent with their occlusion by other regions of the glycoprotein (15). Alterations in the juxtaposition of structures like V3 and C4 that have been implicated in post-receptor binding events may be important to subsequent steps in the virus entry process. Further analysis should provide insight into these issues as well as an understanding of the interaction of neutralizing antibodies with the HIV-1 envelope glycoproteins.

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