Probing the Structure of the Human Immunodeficiency Virus Surface Glycoprotein gp120 with a Panel of Monoclonal Antibodies

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We have probed the structures of monomeric and oligomeric gp120 glycoproteins from the LAI isolate of human immunodeficiency virus type 1 (HIV-1) with a panel of monoclonal antibodies (MAbs); most of these MAbs are directed against continuous epitopes. On native monomeric gp120, most of the first conserved (C1) domain is accessible to MAbs, although some regions of C1 are relatively inaccessible. All of the MAbs directed against the C2, C3, and C5 domains bind preferentially to denatured monomeric gp120, indicating that these regions of gp120 are poorly accessible on the native monomer, although the extreme C terminus in C5 is well exposed. Segments of the V1, V2, and V3 loops are exposed on the surface of monomeric gp120, although the base of the V3 loop is inaccessible. A portion of C4 is also available for MAb binding on monomeric gp120, as is the extreme C terminus in C5. However, on oligomeric gp120-gp41 complexes, only the V2 and V3 loops (and perhaps V1) are well exposed and a segment of the C4 region is partially exposed; continuous epitopes in C1 and C5 that are accessible to antibodies on monomeric gp120 are occluded on the oligomer. Although deletion of the V1, V2, and V3 loops resulted in increased exposure of several discontinuous epitopes overlapping the CD4-binding site, the exposure of most continuous epitopes on the monomeric gp120 glycoprotein was not affected. These results imply a HIV-1 gp120 structure in which the conserved continuous determinants are inaccessible; in some cases, this inaccessibility is due to intramolecular interactions between conserved regions, and in other cases, it is due to intermolecular interactions with other components of the glycoprotein spike. These findings have implications for the design of subunit vaccines based on gp120.

The surface glycoprotein gp120 of human immunodeficiency virus type 1 (HIV-1) attaches the virus to the surface of CD4⁺ cells in the initial stage of the most common route of cellular HIV infection (29, 30, 38). It is therefore an obvious target for antiviral agents aimed at interfering with the HIV infection cycle (33, 44, 79). Furthermore, gp120 is a major target for the host immune response; most of the neutralizing antibody activity in the sera of HIV-1-infected humans is directed against gp120 (4, 49, 58–60, 66, 74, 75).

Both for the rational design of drugs and for the development of future generations of HIV vaccines incorporating gp120, it would be useful to increase our knowledge of gp120 structure. To date, the most informative structural study of gp120 has been the delineation of its disulfide bonding pattern (27). This, together with the definition of variable and conserved gp120 domains (34, 65), provides the fundamental framework for further studies. However, the extensive glycosylation of this molecule, coupled with its conformational flexibility, has so far precluded its crystallization in a form suitable for structural analysis. Furthermore, the lack of any relationship of gp120 to proteins with defined atomic structures, together with its size, renders the generation of realistic computer models unfeasible. We have therefore chosen to map the topology of gp120 and to define the relationships between its domains by immunochemical techniques coupled with site-specific mutagenesis (22, 69–71). Thus, by using

monoclonal antibodies (MAbs) as probes against a panel of gp120 (HxB2) mutants, we have shown, for example, that there is a close spatial relationship between the V3 and C4 regions of gp120 (45, 78).

In the present study, we use a panel of MAbs reactive with continuous epitopes of gp120 to assess which regions of the recombinant, monomeric molecule are exposed on its surface and which are accessible only after denaturation. We find that most of the conserved regions of gp120 are not well exposed on the surface of monomeric gp120. Furthermore, those few conserved continuous elements exposed on the monomeric gp120 glycoprotein (N- and C-terminal regions) are, with a few exceptions, inaccessible on oligomeric gp120 on the surface of infected cells. Thus, there are very few conserved, continuous sites on the native gp120 molecule available for antibody binding, and the well-exposed variable loops can be mutated to destroy antibody epitopes without loss of gp120 function. These results have obvious implications for vaccine design.

MATERIALS AND METHODS

Recombinant gp120 and gp120 peptides. Recombinant gp120 expressed in Chinese hamster ovary cells (batch 42) from the BH10 clone of HIV-1 LAI by Celltech Ltd., Slough, United Kingdom (41) was obtained as purified protein from the reagent repository of the United Kingdom Medical Research Council AIDS Directed Programme (ADP reagent repository). Recombinant gp120 lacking the V1, V2, and V3 loops ($\Delta V1/2/3$) was expressed in *Drosophila melanogaster* Schneider cells under the control of the *Drosophila* metallothionein promoter as previously described (10). The $\Delta V1/2/3$

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plasmid was constructed by substituting the central region of the HxBc2 *env* gene in which the V1, V2, and V3 loops had been deleted (77) for the analogous region of the BH10 *env* gene (the *KpnI*-to-*MstII* fragment) contained in the *Drosophila* expressor plasmid, pMt120delO. The resulting Δ V1/2/3 envelope glycoproteins are chimeric molecules containing BH10 residues at their N and C termini (amino acids 31 to 43 and 365 to 511) and HxBc2 residues in the area spanning the central region from which the V1, V2, and V3 loops had been deleted (77). The Δ V1/2/3 glycoprotein and the wild-type (wt) analog containing the V1, V2, and V3 loops were affinity purified from serum-free culture supernatants of Schneider cells with MAb F105 (56) covalently cross-linked to protein A-Sepharose CL4B (Pharmacia Ltd). The glycoproteins were eluted from the affinity column with 100 mM glycine-HCl, pH 2.8, which was immediately neutralized with Tris base and dialyzed against phosphate-buffered saline (PBS), pH 7.4.

A set of 20-mer gp120 peptides overlapping by 10 amino acids was obtained from the ADP reagent repository (ADP 740). The gp120 sequence used for these peptides corresponds approximately to the BH10 sequence. A separate set of 20-mer peptides from the HxB2R sequence, also overlapping by 10 amino acids but slightly out of phase with the ADP set, was obtained from Cambridge Research Biochemicals (CRB) (Hartston, United Kingdom).

MAbs. All MAbs were murine immunoglobulin G (IgG) except where specified. Some were provided as purified Ig, but most were provided as hybridoma culture supernatants or ascitic fluid samples. Details of MAbs are provided in Results, but references to MAbs previously described elsewhere, together with their donors or commercial suppliers, are as follows: M85, M86, M89, M91 (rat), M92 (rat), and M96 (rat) from Fulvia di Marzo Veronese (12); 7E2/4, 6C4/5, 4D7/4, 8C6/1, and 5F4/1 from Shahin Ranjbar and Harvey Holmes (National Institute of Biological Standards and Control, Pottery Bar, United Kingdom); 133/192, 133/290, 133/237, and 135/9 from Matthias Niedrig (51); B2, B10, B12, B13, B15, B18, B20, B32, C4, C6, C12, and C13 from George Lewis (1); T1.1, L5.1, T9, T7.1, and T2.1 from Lennart Åkerblom, Britta Wahren, and Jorma Hinkula (2, 6); 5B3, 1D10 (rat), and 6D8 (rat) from Philip Berman (47, 48); 7C10, 10A11, 12G10, MF4.1, MF39.1, MF46.1, MF49.1, MF53.1, MF58.1, MF77.1, MF87.1, MF119.1, MF130.1, MF169.1, and MF170.1 from Claudine Bruck (72); W1 and W2 from David Weiner; anti-V1 human polyclonal antibodies from Seth Pincus (54); RSD-33 and antiserum R1/87 from Rod Daniels; G3-4, G3-136, BAT-085 (15, 18, 43, 67) and G3-42, G3-299, G3-508, G3-519, G3-536, and G45-60 (45, 68) from David Ho and Michael Fung; 322-151, 493-156, and 660-178 from Gerard Robey and Mary Pinkus (Abbott Laboratories); 3D3.B8, 4C11.D8 (murine IgM) (6, 52) from Stig Jeansson and Sigvar Olofsson; J1 and J3 from Jim Hoxie and Brad Jameson; 110.C, 110.D, 110.E, 110.H, 110.I, and 110.J from François Traincard (Hybridolabs, Institut Pasteur); 1033-21, 1061-01, and 1044-34 from Jon Laman (23, 24); 9284 and 9301 from Dupont Inc. (64); 110.1 and 110.5 from Genetic Systems Inc. (21, 25); antiserum S1/*env*/9 from David Evans (31, 45); 1C1 from Repligen Inc.; RV110026 from Olympus Inc.; 489.1 (961) from Kate Traynor, National Institute of Allergy and Infectious Diseases AIDS Reagent Program; 4D4#85, 6D5, and 3F5 from Steve Nigida and Larry Arthur; antiserum D7324 from Aalto BioReagents; and 15e, F-91, and 21h from James Robinson and David Ho (19, 69). In addition, MAbs obtained from the ADP reagent repository via the manager, Harvey Holmes, and their original donors were as follows: ADP 327, ADP 329, ADP 332, and ADP 334 from Claudine Bruck (72);

ADP 323 and ADP 326 from Mark Page; ADP 301 from Rod Daniels; ADP 360 from Richard Tedder; ADP 388 (rat) and ADP 389 (rat) from Chris Dean (9); and ADP 3028 from B. Vestegaard and E. Hulgaard.

The immunogens used to derive the above MAbs are described in the primary references, but note that the majority were raised against recombinant or virus-derived gp120 or gp160 preparations of predominantly native configuration. The actual state of folding of the immunogenic molecule for each MAb is, of course, unknown. Those MAbs donated by George Lewis were raised against gp160 from MicroGeneSys Inc., which is a misfolded protein (1, 40), and the MAbs from Jon Laman and from Jim Hoxie and Brad Jameson were antipeptide antibodies. These MAbs are starred in Tables 1 to 4.

Reactivities of MAbs with solid-phase peptides. Ascertaining the reactivities of MAbs with solid-phase peptides was performed essentially as described previously (36, 43, 45). Peptides were adsorbed at 10 μ g/ml in Tris-buffered saline (TBS) pH 7.5, onto Immulon II enzyme-linked immunosorbent assay (ELISA) plates (Dynatech Ltd.). After unbound peptides were washed away and the plates were blocked for 30 min with a solution of 2% nonfat milk powder in TBS, MAbs in TMSS buffer (TBS containing 2% nonfat milk powder and 20% sheep serum) plus 0.5% Tween 20 were added to the plates. The MAb concentrations tested were those that gave maximum binding to denatured gp120 in the absence of significant assay background. For some MAbs, Tween 20 was omitted from the reaction buffer. Bound MAb was detected as described previously (35, 36, 39).

Only peptides giving significant (optical density at 492 nm [OD_{492}] > 0.100) reactivity with a MAb are recorded in the tables. At the minimum, each MAb was tested for binding to the three peptides flanking the most reactive peptide and usually on two sets of peptides. If a MAb is reactive with only one peptide (e.g., the peptide containing amino acids 91 to 110 [91-110]), its binding site is probably within the central 10 amino acids of the peptide (amino acids 95 to 105); a MAb reactive with two adjacent peptides (e.g., peptides 91-110 and 101-120) probably binds within the overlapping amino acids (101 to 110). A few C1 MAbs also react significantly with peptides from the C2 region of gp120; such reactivities are not recorded in this report, because their importance is under investigation. Many of the C1, C2, and C3 MAbs are currently being analyzed with nonamer peptides overlapping by eight amino acids; to date, we have not noticed any major discrepancies from the data obtained with the above 20-mer peptides (28).

Reactivities of MAbs with native and denatured monomeric gp120. The reactivities of MAbs with native and denatured monomeric gp120 was determined essentially as described elsewhere (37, 45). Briefly, BH10 gp120 (25 ng/ml) was captured onto a solid phase via its carboxy terminus by using sheep polyclonal antibody D7324. The gp120 glycoprotein was added either in TBS containing 10% fetal calf serum (TBS-FCS) or in the same buffer plus 1% Nonidet P-40 nonionic detergent (TBS-NP-40). Alternatively, gp120 at 250 ng/ml was denatured by boiling for 5 min in TBS-FCS plus 1% sodium dodecyl sulfate (SDS) and 50 mM dithiothreitol (DTT) before dilution 10-fold into TBS-NP-40. SDS was not necessary for gp120 denaturation and exposure of buried epitopes but was routinely included. Antibody D7324 reacts equivalently with native and denatured gp120. MAbs were added to gp120 in TMSS buffer and further supplemented with 0.5% Tween 20 nonionic detergent for experiments on NP-40-treated or denatured gp120.

For analysis of MAb epitopes in the V5 and C5 domains

close to the carboxy-terminal capture antibody, we also used a complementary procedure. gp120 (50 ng/ml) was solubilized in TBS-NP-40 with or without prior denaturation with SDS-DTT as described above and then incubated with a range of MAb concentrations for 0.5 h before addition of the complexes to wells containing adsorbed rabbit polyclonal anti-gp120 antiserum R1/87 (39). Bound MAb was detected as outlined above, except that 10% FCS was used instead of sheep serum.

The reactivities of some MAbs with wt HxBc2 gp120 and Δ V1/2/3 mutant glycoproteins were compared directly by using either native proteins or proteins denatured with SDS-DTT. To ensure loading of the solid phase with equivalent amounts of the wt and Δ V1/2/3 proteins, these were titrated and bound gp120 was detected with MAb 133/290 directed against a continuous C1 epitope (51) whose exposure was only minimally affected by gp120 denaturation. The amounts of wt and Δ V1/2/3 proteins giving equivalent reactivities with MAb 133/290 were then used in subsequent experiments with other MAbs.

Binding of MAbs to oligomeric gp120: flow cytometry. H9 cells were maintained in RPMI 1640 medium with 10% FCS. When semiconfluent, 10^7 cells were transfected with 10 μ g of the HIV-1 molecular clone pHxB2 by the standard DEAE-dextran procedure. The cells were cultured for 7 days, after which there was no detectable CD4 present on the cell surface and no further cytopathic effect. We had previously observed that HIV-1 envelope glycoprotein expression was maximal between 1 and 4 weeks after transfection (42, 61, 62), so all experiments were carried out during this period.

Cells were washed twice in growth medium and resuspended at 6×10^6 cells per ml in ice-cold wash buffer (PBS–1% FCS–0.02% sodium azide). MAbs were titrated in round-bottomed wells of a microtiter plate in 25- μ l portions of wash buffer, and then 25- μ l portions of cell suspension were added. After incubation for 2 h on ice or at 37°C, the cells were washed three times in cold wash buffer, and phycoerythrin-labelled anti-mouse Ig or anti-rat Ig (Immunotech, Luminy, France) in a total volume of 50 μ l was added to the cell pellet in each well. After incubation for 30 min on ice, the cells were washed as described above, resuspended in 200 μ l of PBS–1% FCS–1% formaldehyde per well, and stored overnight at 4°C. The fixed cells were analyzed by flow cytometry on a Becton Dickinson FACScan (BDIF, San Jose, Calif.) with Consort 30 software. Results are presented as the mean fluorescence intensities of the test samples minus the background staining with the fluorochrome conjugate alone.

Mapping MAb epitopes with gp120 mutants. The production of HxBc2 gp120 mutants and their use in mapping antibody epitopes has been described previously (67, 69–71). Soluble gp120 molecules secreted from transfected COS cells were used to map MAb epitopes on native gp120 in an immunoassay format exactly as described elsewhere (43, 45). For mapping MAb epitopes on denatured gp120, the transfected COS cells containing gp120 and gp160 were pelleted and lysed in radioimmunoprecipitation assay buffer (69–71). The lysates were then treated with 1% SDS and 50 mM DTT and boiled for 5 min before 10-fold dilution into TBS-NP-40. The diluted lysates were stored at –20°C and used as a stock of denatured gp120 or gp160 mutants. Mapping MAb epitopes on denatured gp120 or gp160 was performed exactly as described for native gp120, except that the MAb-gp120 or gp160 reaction buffer contained 0.5% Tween 20. However, instead of using HIV-1⁺ serum to normalize for variable gp120 concentrations, we used a MAb, 9301, directed against a continuous epitope (residues 475 to 485) in the C5 region. The normalization procedure was otherwise as described for native gp120

(43, 45), save that gp120 mutants with substituted amino acids within the 9301 MAb epitope were excluded from the analysis. In the present report, only mutants close to the peptide epitope that show reduced MAb binding are recorded. Under native conditions, the binding of several MAbs to continuous epitopes in one domain is increased or decreased by distant-site mutations in a separate domain. These effects are under investigation and will be reported elsewhere.

The gp120 mutants used in these experiments were as follows: 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, Δ 119-205, 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. Some MAbs were tested against several additional mutants; these are listed in the text if they showed a significant alteration of binding compared with that of wild-type gp120.

RESULTS

Definition of MAb epitopes. To characterize a MAb, we initially assessed its binding to native and SDS-DTT-denatured BH10 gp120 glycoproteins. Any MAb significantly reactive with denatured gp120 (see below) was presumed to be able to bind a peptide. We localized the epitopes for these MAbs to within 10 to 20 amino acids in solid-phase binding assays using two sets of LAI-derived 20-mer peptides with a 10-amino-acid overlap. Usually, qualitatively similar results were obtained with the two sets. For many MAbs, the epitopes were confirmed with a panel of HxB2 gp120 mutants containing single or a few amino acid substitutions. These mutants were used as native proteins or after SDS-DTT denaturation as appropriate. Details of the epitopes for individual MAbs are tabulated below (see Tables 1 to 4).

Antibody-accessible and -inaccessible regions of monomeric gp120. To ascertain whether a MAb epitope was readily accessible on the surface of monomeric gp120, we determined whether the MAb reacted preferentially with recombinant BH10 gp120 in the absence of detergent or after denaturation of the molecule by boiling in the presence of SDS and DTT. Data for individual MAbs are recorded in Tables 1 to 4; values for the fold increase in binding to denatured gp120 relative to native gp120 represent the means of from two to six determinations. In Fig. 1 we demonstrate our criteria for deciding the status of a MAb epitope.

Some MAbs bind negligibly to native gp120 but bind strongly once gp120 is denatured, exemplified by the C2 MAb 322-151 and the C3 MAb B32 (Fig. 1a and b). We interpret this to mean that the MAb epitope is continuous and is located, at least in part, in the interior of the protein. In all such cases, the MAb reacted efficiently with one or more peptides corresponding to a particular gp120 segment (Tables 1 to 4). In contrast, other MAbs such as the human MAb 15e bind only to native gp120 (Fig. 1c), because their epitopes are discontinuous (19, 69). Another set of MAbs, represented by the C1 MAb 133/237, bind with approximately equal affinities to native and denatured gp120 (Fig. 1d) and are peptide reactive. A few other peptide-reactive MAbs show a modest reduction in binding to denatured gp120 compared with that of native gp120 (data not shown). The epitopes for both these sets of

FIG. 1. Reactivities of MABs with native and denatured monomeric gp120 glycoproteins. MABs at the concentrations or dilutions indicated were reacted with native (○) or SDS-DTT-denatured (●) recombinant BH10 gp120 before determination of gp120-bound antibody. All data are corrected for background antibody absorption in the absence of gp120 (usually <0.100 OD₄₉₂).

MABs are assumed to be continuous regions of gp120 exposed on the surface of the protein.

The most common type of MABs in our panel are able to bind to native gp120 but bind significantly more avidly to denatured gp120 and are peptide reactive, exemplified by the C1 MABs T7.1 and 12G10 (Fig. 1e and f). When interpreting these observations, the following factors were considered. First, a continuous peptide epitope of between 5 and 10 amino acids may not be fully exposed on the surface of the native protein; occlusion by adjacent polypeptide regions or carbohydrates may reduce accessibility of the MAB to part of its epitope. This may be complicated further by molecular flexibility. Second, a preparation of recombinant gp120 is not homogenous; it may contain fully folded, partly denatured, and completely unfolded species (20), and it may undergo further modification *in vivo*, for example, by partial proteolytic degradation. Thus, the conformation of the immunogen for a particular antibody may not necessarily reflect that of the correctly folded gp120 molecule. The batch of BH10 gp120

expressed in CHO cells that Kaufmann et al. used contained 50.3% native molecules, judged by their ability to bind CD4 (20). We assume that our batch is of comparable quality; thus, about half of the gp120 molecules could contain some inappropriately exposed surface elements. Taking all these factors into consideration, we judge that a fivefold increase in the affinity of a MAB for denatured gp120 compared with that for native gp120 (i.e., a leftward shift of fivefold in the titration curves) indicates that the epitope is inaccessible on the surface of the native molecule. Obviously, the greater the relative increase in binding on denaturation, the less exposed the epitope. Thus, we assess the epitope for MAB T7.1 (Fig. 1e) to be in a region of gp120 partially exposed on the surface and that for 12G10 (Fig. 1f) to be in an internal, mostly inaccessible region. MABs from each conserved domain of gp120 were tested on both recombinant HxB2 and BH10 gp120 glycoproteins with and without denaturation; very few significant differences in epitope exposure were noted when one protein was used in place of the other (data not shown).

To address whether weak binding of some MABs to native recombinant BH10 gp120 did indeed reflect the presence of some inappropriately folded molecules, we compared MAB reactivity against recombinant gp120 to reactivity against gp120 obtained by disrupting HxB2 virus-containing culture supernatants with NP-40 nonionic detergent. This process provided an alternative source of gp120 that had been synthesized by a more natural route and that had not been subjected to purification procedures. First, we determined for each MAB the effect of the nonionic detergent NP-40 on its binding to recombinant gp120. The addition of NP-40 did not increase the binding of any MAB that reacted preferentially with the denatured protein. Thus, nonionic detergent was not sufficient to cause exposure of regions of gp120 we assess as internal (data not shown). The binding of most of the MABs apparently reactive with surface-exposed continuous epitopes was not increased significantly by NP-40. In fact, the binding of some MABs to the C1 region from 80-110, the V4 region 395-400, and the C4 region 430-440 was moderately decreased by NP-40. In contrast, the binding of MABs to the V2 and V3 loops and to discontinuous gp120 epitopes was usually increased 3- to 30-fold by treatment of gp120 with NP-40 (data not shown).

When we compared how selected MABs directed against apparently inaccessible segments of each of the conserved regions reacted with recombinant and virus-derived gp120 in the presence of NP-40 detergent, we observed that virus-derived gp120 always had higher binding affinities for these MABs. Indeed, the affinities of these MABs for virus gp120 were typically 4- to 10-fold lower than for the recombinant gp120 (data not shown). Thus, a minor proportion of the recombinant molecules are probably misfolded and expose several conserved regions inappropriately. Hence some MAB epitopes that we assess as buried are likely to be even less exposed on correctly folded monomeric gp120 than indicated by the data in Tables 1 to 4 and Fig. 1.

Effects of deletion of the V1, V2, and V3 loops on exposure of gp120 conserved regions. We have noted that several MABs directed against discontinuous epitopes overlapping the CD4-binding site bind with up to a 50-fold-higher affinity to a mutant HIV-1 envelope glycoprotein lacking the V1, V2, and V3 loops than to the wt envelope glycoprotein (77). These experiments were performed with the multimeric envelope glycoprotein complex expressed on cell surfaces. To address whether some of the conserved regions of monomeric gp120 might be hidden under the variable loops, we measured the binding of selected MABs to HxB2 gp120 and the Δ V1/2/3

FIG. 2. Reactivities of MAbs with native and denatured HxB2 and Δ V1/2/3 gp120 glycoproteins. MAbs at the concentrations indicated were reacted with native (open symbols) or SDS-DTT-denatured (closed symbols) recombinant HxB2 gp120 ($\circ$, $\bullet$) or the Δ V1/2/3 mutant ($\square$, $\blacksquare$) before determination of gp120-bound antibody. Background antibody absorption in the absence of gp120 ($\triangle$).

mutant before and after denaturation (Fig. 2). Data for individual MAbs are tabulated in Tables 1 to 4.

We normalized loading of the solid phase with equal amounts of the wt and Δ V1/2/3 proteins by determining the concentrations that gave equivalent binding of MAb 133/290 directed against the continuous C1 epitope represented by amino acids 62 to 71 (51). Binding of this MAb to both forms of gp120 was unaffected by denaturation (Fig. 2a). In contrast, human MAb 21h directed against a discontinuous gp120 epitope overlapping the CD4-binding site (19, 69) did not bind to denatured gp120 and bound with about 12-fold-higher affinity to the Δ V1/2/3 mutant than to the wt gp120 (Fig. 2a). Similar results were obtained with other MAbs that also overlap the CD4-binding site, for example, 15e (19), F-91, ADP 326 (CRA-5), and ICR39.13g (9) (data not shown). This finding demonstrates that the major variable loops can mask some of the discontinuous, conserved neutralization epitopes on the monomeric gp120 glycoprotein, as has been observed for the oligomeric envelope glycoprotein complex (77).

MAb G3-299, recognizing a conformationally sensitive C4 epitope that is also dependent upon V3 sequences (45), failed

to bind the Δ V1/2/3 mutant, probably because of its lack of the V3 loop (Fig. 2c). A second C4 MAb, G45-60 (45, 68), showed a modest reduction in binding to either form of denatured HxB2 gp120 but was not affected by deletion of the variable loops (Fig. 2d). As found with BH10 gp120 (Fig. 1), most MAbs directed against continuous epitopes in the conserved regions bound with increased affinities to denatured wt and Δ V1/2/3 HxB2 gp120, exemplified by the C1 MAb T9 and the C5 MAb 1C1 (Fig. 2e and f). The T9 epitope is partially exposed on native wt gp120 and the 1C1 epitope is poorly exposed, as judged by the 4- and 17-fold increases in binding of these MAbs after gp120 denaturation, respectively. Neither of these MAbs showed a significant increase in binding to the native Δ V1/2/3 molecule compared with that of wt gp120. This was also the case for all the other MAbs we evaluated (see tables 1 to 4). Thus, we conclude that occlusion of the conserved, continuous regions of gp120 is not dependent upon the V1, V2, and V3 variable loops.

Epitope exposure in the C1 domain. The extreme amino terminus of monomeric gp120 (amino acids 31 to 40) is well exposed for MAb binding, exemplified by MAbs 7E2/4 and M85 (Table 1). Both these MAbs react relatively poorly with denatured gp120, suggesting a conformational component to their epitope, yet both react with peptides spanning amino acids 31 to 50. However, these two MAbs are unique in our experience, in that they are strongly peptide reactive only when nonionic detergent is omitted from the binding reaction; most MAbs show increased binding in the presence of nonionic detergents such as Tween 20. The gp120 stretch from approximately amino acids 41 to 55 is also accessible on the protein's surface for binding of MAbs M92, M86, and 4D4#85, as is the segment from amino acids 61 to 70 recognized by MAbs 133/237 and 133/290. Residues 81 to 90 (MAb ADP 360) are not well exposed on the native protein, nor are amino acids 85 to 95 (approximate epitopes for MAbs 1D10 and T9). This may partially reflect a local influence of the carbohydrates on Asn-88, because removal of the glycosylation site in the 88N/P mutant leads to a modest increase in the binding of T9 (data not shown). However, neither ADP 360 nor 1D10 binds to the 88N/P mutant, implicating Asn-88 as a component of their epitopes (Table 1).

Several MAbs reactive with peptide 91-100 bind about as well to native gp120 as to denatured gp120; these MAbs include 133/192, C6, B2, B10, MF49.1, and 489.1 (Table 1). However, other MAbs reactive with the same peptides (T1.1, T7.1, T9, and 5B3) bind significantly better to denatured gp120 than to the native molecule. Of these, 5B3 has a high affinity for native gp120 (data not shown) and has been shown elsewhere to be able to block the binding of monomeric gp120 to CD4 (47). It also binds to oligomeric gp120 (see Table 5). Thus, the 91-100 segment of C1 is likely to be well exposed on the protein's surface, but the epitopes for some MAbs to this region are not optimally accessible on the folded protein (76). Each of the epitope 91-100-reactive MAbs binds poorly or not at all to the ADP peptide containing Asp-94 but binds strongly to the CRB peptide with Asn-94, implicating this residue in their epitopes.

MAbs reactive with the segment from peptide 101-110 (MF49.1, B18, and B20) bind equivalently to denatured and native gp120, indicating that most of this region is exposed. All but one of the MAbs reactive only or most strongly with peptide 101-120 and, in some cases, weakly reactive with peptide 111-130, bind 6- to 30-fold more strongly to denatured gp120 than to native gp120. MAb 6D5 reactive with peptide 122-141 from HxB2 and able to bind strongly to denatured BH10 gp120 (Table 1) was raised against MN gp120. Because

TABLE 1. MAb epitope exposure in the gp120 C1 domain

MAb ^a	Reference ^b	MAb-peptide reactivity		HxB2 gp120 mutant ^c with impaired binding to MAb		Relative affinity ^d of MAb for:	
		Peptide	OD ₄₉₂	Native gp120	Denatured gp120	Denatured gp120 ^e	$\Delta V_{1/2/3}$ ^f
7E2/4	12	31-50	0.255	36V/L		0.07	3.2
M85		30-51	1.134	36V/L 40Y/D		<0.01	
M92	12	31-50	1.309			1.0	0.3
4D4#85		41-60	1.673				
		31-50	0.204	36V/L			
M86	12	41-60	1.391	45W/S		0.10	
		42-61	1.818			1.0	
133/237	51	51-70	0.731	69W/L		1.4	
133/290	51	61-80	0.948				
		51-70	0.801	69W/L	69W/L	2.2	0.8
4A7C6 (ADP 360)		61-80	0.627				
		71-90	1.765	88N/P	88N/P	7.9	0.3
1D10	5	81-100	1.907				
133/192	51	81-100	1.290		88N/P	13	
		81-100	1.606	None		1.8	2.2
C6*	1	91-110	0.712				
		81-100	1.887			0.9	
B2*	1	91-110	1.790				
		81-100	1.990	103Q/F	None	1.4	
B10*	1	91-110	1.579	106E/A			
		81-100	1.284			0.4	
489.1 (961)		91-110	1.299				
		81-100	1.574			1.0	
T1.1	2	91-110	1.687				
		81-100	1.502		None	1.0	
T7.1	6	91-110	1.574				
		81-100	1.189		None	4.0	
T9	6	91-110	1.126				
		81-100	1.067	102E/L	None	7.9	0.4
5B3	47	91-110	0.625	103Q/F			
		81-100	1.333	None	113D/A	8.3	
MF49.1	72	91-110	1.165		113D/R		
		81-100	1.324		117K/W		
B20*	1	91-110	1.467		None	3.8	
		101-120	1.806				
B18*	1	91-110	1.805			1.0	
		101-120	1.822				
MF39.1	72	91-110	1.798			1.0	
		101-120	1.793				
T2.1	6	91-110	0.345		102E/L	30	
		101-120	1.350		113D/R		
6D8	5	101-120	1.818	113D/R	102E/L	0.27	
					106E/A		
W1					113D/R		
					117K/W		
M96	72				102E/L	15	
					106E/A		
					113D/R		
					113D/A		
					117K/W	6	
					103Q/F		
					113D/A		
					113D/R		
					117K/W		
M96	72	101-120	1.788	103Q/F		6	3.1
				106E/A			
				113D/A, 113D/R, 117K/W			

Continued on following page

TABLE 1—Continued

MAb ^a	Reference ^b	MAb-peptide reactivity		HxB2 gp120 mutant ^c with impaired binding to MAb		Relative affinity ^d of MAb for:	
		Peptide	OD ₄₉₂	Native gp120	Denatured gp120	Denatured gp120 ^e	$\Delta V1/2/3^f$
37.1.1 (ADP 327)	72	101-120	1.706		102E/L 113D/A 113D/R 117K/W	8.6	1.4
187.2.1 (ADP 332)	72	101-120	1.499		102E/L 113D/A 113D/R 117K/W	7	1.4
MF58.1	72	101-120	1.505		102E/L 106E/A 113D/A 113D/R 117K/W	10	
MF77.1	72	101-120	1.927		113D/A	11	
MF119.1	72	101-120	2.059	102E/L 113D/A 113D/R 117K/W	113D/R 117K/W 113D/A 113D/R 117K/W	30	
MF4.1	72	101-120	1.693			8	
MF53.1	72	101-120	1.756			10	
135/9	51	101-120	1.833	113D/A	102E/L	15	2.0
		111-130	0.230	113D/R	106E/A 113D/A 113D/R 117K/W		
MF46.1	72	101-120	1.441		113D/A	8.5	
		111-130	0.245		113D/R 117K/W		
C4	1	101-120	1.457		113D/A	10	
		111-130	0.280		113D/R		
10A11	72	101-120	1.681		113D/A	7.8	
		111-130	0.203		113D/R 117K/W		
12G10	72	101-120	1.593		113D/A	17	
		111-130	0.123		113D/R 117K/W		
7C10	72	101-120	1.816		102E/L	5.8	
		111-130	0.200		103Q/F 113D/A 113D/R 117K/W		
6D5		122-141	1.050		117K/W 120/121VK/LE Δ119-205 125L/G	15	

^a The starred MAbs, C6, B2, B10, B18, and B20, were raised against a misfolded form of gp160 (1, 40); the immunogens for all the other MAbs were envelope glycoprotein preparations that contain predominantly correctly folded molecules.

^b Citation for original description of MAb; donors of unpublished MAbs are listed in Materials and Methods.

^c Mutants highlighted by bold type show very weak or no binding.

^d Measured in fold increase in binding.

^e SDS-DTT-denatured gp120 compared with native gp120.

^f $\Delta V1/2/3$ mutant compared with wt HxB2 gp120. A blank entry indicates that the MAb was not tested.

the V1 segment from amino acids 127 to 141 is very variable between isolates (46), it seems likely that the epitope for 6D5 lies within the C1 segment spanning residues 122-126; this region is not exposed on the native molecule.

Thus, we conclude that much of the gp120 segment from approximately amino acids 110 to 126 is inaccessible on the native protein. However, MAb T2.1, while clearly reactive with peptide 101-120, is sensitive to gp120 conformation and binds

poorly to the denatured molecule (Table 1). Binding of MAb T2.1 to native gp120 is also almost completely abolished by nonionic detergent (data not shown), an unusual property in our experience. Part of the 110-120 region is probably antibody accessible, but our mapping procedures lack the resolution to define precisely how much. The sensitivity of MAb T2.1 to the 113D/R substitution (Table 1) might suggest that this residue is partly accessible, consistent with its apparent role in some

discontinuous MAb epitopes (69–71). Fine mapping of the peptide epitopes for many of the above C1 MAbs is in progress (28).

Epitope exposure in the V1 and V2 domains. We know of no MAbs against the V1 domain of gp120 from any isolate. However, serum from a laboratory worker infected with the HIV-1 IIIB virus reacts strongly with HxB2 peptides 131–150 and 141–160, the ELISA OD₄₉₂ values being 0.694 and 1.154, respectively, at a serum dilution of 1:1,000 (43, 55). Polyclonal serum antibodies affinity purified from this individual with an V1 peptide are able to bind to native gp120 at least as strongly as to denatured gp120 (55). Furthermore, these V1 antibodies weakly neutralize HIV-1 IIIB (54). Thus, a region at the tip of the V1 loop within approximately amino acids 140 to 150 is a surface-exposed epitope on monomeric gp120 and presumably on the oligomer.

We have described elsewhere details of epitope exposure in the V2 region of gp120 (43). In summary, two MAbs RSD-33 and 6C4/5 were reactive with peptides 152–171 and 162–181, indicating that their epitope is likely to be between amino acids 162 and 171. Both MAbs bound weakly to native BH10 gp120, and their affinities were increased by approximately fourfold when gp120 was denatured (43). These results suggest that residues 162 to 171 in the amino-terminal flank of the V2 loop are no more than partially accessible for MAb binding on the native gp120 monomer (see Fig. 4). McKeating et al. (32) have suggested that residues 162 to 170 constitute a neutralization epitope, implying exposure of this region on the native glycoprotein. Since we have not been able to test these MAbs in our assays, the status of the amino-terminal flank of V2 remains uncertain.

The crown of the V2 loop is, in contrast, a well-characterized neutralization epitope (15, 18, 43, 67, 80). This is exemplified by MAb BAT-085 which binds strongly to a peptide spanning amino acids 169 to 180 and recognizes native monomeric and oligomeric gp120 glycoproteins (15, 43). It should, however, be noted that most V2-sensitive MAbs are not strongly peptide reactive (15, 18, 43, 67). Thus, we conclude that the crown of the V2 loop is well exposed on the native glycoprotein but the amino-terminal flank may or may not be. This conclusion is supported by studies on the V2 loop of simian immunodeficiency virus (SIV); MAb KK13 directed against the central region of the SIV V2 loop is potently neutralizing, whereas MAb Senv1.1 directed against the amino-terminal flank of SIV V2 is not (14).

Epitope exposure in the C2 domain. MAbs raised against gp120 that recognize the C2 region fall into two clusters: those reactive with epitope 201–230 and those binding within epitope 250 and 280 (Table 2). In addition, two anti-peptide MAbs J1 and J3 bind within region 222–231. Thus, we have no information on the exposure of about half of the C2 region. The data that we do have indicate that the immunogenic regions of C2 are poorly accessible on gp120's surface. None of the six MAbs mapping within approximately amino acids 210 to 230 nor any of the nine MAbs recognizing the 250–280 region bound well to native gp120. Four MAbs, MF87.1, MF169.1, MF170.1, and 213.1, bound strongly to peptides 242–261 and 252–271 in the ADP set but bound only to peptide 251–270 in the CRB set (MAb MF170.1 bound weakly to peptide 241–260 from this set). Thus, the core epitope for these MAbs is region 252–261, with the presence of Leu-261 being critical. MAb 110.C directed against peptide 271–280 (fine mapped to 277–279 [73]) bound about as well to native gp120 as to denatured gp120 (Table 2), but its affinity was very low. Thus, it is possible that the region near amino acid 280 is exposed on the surface of gp120 but perhaps occluded by the carbohydrates on Asn-276.

Epitope exposure in the V3 domain. It is well established that the V3 loop is a region on the surface of gp120 that is important for HIV neutralization. However, not all of the V3 region is exposed for antibody binding. We have shown elsewhere that anti-peptide MAbs reactive with the peptides 294–300 (INCTRPN) and 320–328 (IGKIGNMRQ) at the amino-terminal base and on the carboxy-terminal flank, respectively, are essentially unreactive with native monomeric gp120 but bind to the denatured molecule or to detergent-treated gp120 (24). Thus the V3 flanks and base are relatively inaccessible when gp120 is folded. In contrast, the central region of V3 is well exposed; this is exemplified by MAbs 9284, 110.5, and 110.I, which bind 30-, 50-, and 20-fold more strongly to native BH10 gp120 than to denatured gp120, respectively (data not shown). The peptide epitopes for these MAbs have been partially or completely defined as regions 301–312 (64), 309–317 (25), and 316–322 (73). Thus, the V3 segment from approximately amino acids 301 to 322 is antibody accessible on monomeric gp120, but the segments on either side of this central sequence are not. Precisely how much of the amino-terminal flank is exposed is not certain; we suspect that the transition from buried to exposed occurs around amino acid Thr-303.

Epitope exposure in the C3 domain. Few MAbs directed against the C3 domain have been isolated; each of the five we have tested reacts with peptides from the 360–393 region (Table 3). Thus, we have no information on the exposure of the C3 region from the base of the V3 loop at residue 330 to approximately residue 365. It is clear, however, that the 360–393 region is completely inaccessible to MAbs on the native BH10 gp120 molecule. None of the five MAbs available binds significantly to native gp120, but each reacts strongly with the denatured molecule, exemplified by MAb B32 (Fig. 1b). Thus, amino acids Asp-368 and Glu-370, critical for binding of CD4 and many CD4-blocking MAbs (53, 69, 71) are contained within a region of gp120 that is relatively inaccessible as a continuous domain.

Epitope exposure in the V4 and C4 domains. Epitope exposure in V4 and C4 has been discussed extensively elsewhere (45) and will only be summarized here. One nonneutralizing MAb, B15, directed against the V4 domain. B15 bound strongly to peptide 392–411 of the ADP peptide set (OD₄₉₂ of 1.899) but did not bind to the flanking peptide 380–393 or 402–420. The CRB peptides most strongly reactive with B15 were 381–400 and 391–410, with OD₄₉₂ values of 1.939 and 2.004, respectively. Fine peptide scanning with nonamer peptides indicate that the B15 epitope is between amino acids 395 and 400 (28). The binding of B15 to both native and denatured gp120 glycoproteins was destroyed by the 395W/S mutation and was reduced by the 386N/R change. There was a 5-fold increase in B15 binding to BH10 gp120 on denaturation, and a 2.5-fold decrease in binding to the ΔV1/2/3 mutant compared with that of wt HxB2 gp120. A second V4 MAb, ADP3028 (2D7/V4), reacted strongly with peptides 392–411 (OD₄₉₂ of 1.423), 381–400 (OD₄₉₂ of 1.228), and 391–410 (OD₄₉₂ of 1.384), localizing its epitope to residues 392 to 400. This MAb was reactive only with denatured gp120. Thus, this stretch of V4 is poorly exposed on the surface of gp120. The local carbohydrates on asparagine residues 392, 397, and 406 could contribute to the masking of this area.

The C4 region corresponding approximately to residues 430 to 438 is well exposed on native monomeric gp120 (45). In addition, part of the segment from region 423–430 is accessible to neutralizing MAbs (5, 48). An anti-peptide serum to epitope 436–450 bound preferentially to denatured gp120, indicating that the C-terminal part of C4 is poorly accessible on the native

TABLE 2. MAb epitope exposure in the gp120 C2 domain

MAb ^a	Reference ^b	MAb-peptide reactivity		HxB2 gp120 mutant ^c with impaired binding to MAb		Relative affinity ^d of MAb for:	
		Peptide	OD ₄₉₂	Native gp120	Denatured gp120 ^e	Denatured gp120 ^f	$\Delta V_{1/2/3}$ ^g
3D3.B8	6	201-220	0.176			≥ 10	
		211-230	0.269				
4C11.D8	6	201-220	0.798			≥ 10	
		211-230	0.470				
322-151		201-220	0.226		207K/W	30	0.8
		211-230	1.486				
493-156		211-230	0.753			> 10	
J1*		212-231	1.396		None	30	1.3
		222-241	1.448				
J3*		212-231	1.236		None	30	
		222-241	1.256				
MF87.1	72	242-261	1.659		252R/W	10	
		252-271	1.650		257T/G		
					257T/R		
					267E/L		
					269E/L		
MF169.1	72	242-261	1.762		252R/W	11	0
		252-271	1.718		257T/R		
					257T/G		
					267E/L		
MF170.1	72	242-261	1.731	252R/W	252R/W	15	0.25
		252-271	1.629	257T/R	257T/R		
				257T/G	257T/G		
				262N/T	267E/L		
				269E/L			
				281A/V			
213.1 (ADP 334)	72	242-261	1.542		252R/W	100	
		252-271	1.370		257T/R		
					257T/G		
					266A/E		
					267E/L		
					269E/L		
M89	12	252-271	1.383		252R/W	> 30	No binding ^h
					257T/R		
					269E/L		
					269E/L		
B13*	1	252-271	1.800		252R/W	30	
					257T/R		
					257T/G		
					267E/L		
					269E/L		
B12*	1	252-271	1.867		257T/R	27	
					262N/T		
					267E/L		
					269E/L		
C13*	1	252-271	1.447		252R/W	36	0.05
					262N/T		
					267E/L		
					269E/L		
110.E	73	262-281	1.915			7.3	No binding
110.C	73	261-280	1.075			1.0	No binding
		271-290	1.640				

^a The starred MAbs B12, B13, and C13 were raised against a misfolded form of gp160 (1, 40), and starred MAbs J1 and J3 are anti-peptide MAbs; the immunogens for all the other MAbs were envelope glycoprotein preparations that contain predominantly correctly folded molecules.

^b Citation for original description of MAb; donors of unpublished MAbs are listed in Materials and Methods.

^c Mutants highlighted by bold type show very weak or no binding.

^d Measured in fold increase in binding.

^e Note that except for MAb B12, the 262N/T mutant was not in the test panel.

^f SDS-DTT-denatured gp120 compared with native gp120.

^g $\Delta V_{1/2/3}$ mutant compared with wt HxB2 gp120. A blank entry indicates that the MAb was not tested.

^h The MAb was unreactive with the native wild-type HxB2 molecule.

TABLE 3. MAb epitope exposure in the gp120 C3 domain

MAb ^a	Reference ^b	MAb-peptide reactivity		Denatured gp120 mutant ^c with impaired binding to MAb	Relative affinity ^d of MAb for:	
		Peptide	OD ₄₉₂		Denatured gp120 ^e	$\Delta V1/2/3^f$
4D7/4	72	361-380	1.931	380G/F 381E/P 386N/R 392N/R + 397N/R	≥ 10	No binding ^g
36.1 (ADP 329)		362-381	1.864		>30	
C12*	1	362-381	0.705	380G/F 381E/P 384Y/E 386N/R 392N/R + 397N/R	>30	No binding
		380-393	0.330			
110.D	73	380-393	1.777	380G/F 381G/P 382F/L 384Y/E 386N/R	>50	
B32*	1	380-393	1.682		>100	

^a The starred MAbs C12 and B32 were raised against a misfolded form of gp160 (1, 40); the immunogens for all the other MAbs were envelope glycoprotein preparations that contain predominantly correctly folded molecules.

^b Citation for original description of MAb; donors of unpublished MAbs are listed in Materials and Methods.

^c Mutants highlighted by bold type show very weak or no binding.

^d Measured in fold increase in binding.

^e SDS-DTT-denatured gp120 compared with native gp120.

^f $\Delta V1/2/3$ mutant compared with wt HxB2 gp120. A blank entry indicates that the MAb was not tested.

^g The MAb was unreactive with the native wild-type HxB2 molecule.

molecule (45). No reagents are available to probe the stretch from amino acids 450 to 460.

Epitope exposure in the V5 and C5 domains. The V5 and C5 regions from approximately amino acids 460 to 500 are very poorly accessible on monomeric gp120 (Table 4). All the MAbs mapping to V5 (M91, 460-470) or C5 (CRA1, 465-475; eight MAbs, 475-485; RV110026, 490-500) bind 10- to 30-fold more strongly to denatured gp120 than to native gp120. We confirmed that this was not an artifact of the use of capture antibody D7324 to C5 epitope 497-511 by substituting a rabbit polyclonal anti-gp120 antibody; the revised assay gave qualitatively similar results (Table 4). Some of the MAbs directed against region 475-485 (notably 1C1) do bind quite well to native monomeric gp120; a small part of this region might be antibody accessible. Finally, the extreme carboxy terminus of gp120, approximately amino acids 500 to 511, is well exposed on monomeric gp120, as evidenced both by the results obtained with MAb 110.1 (Table 4) and our routine use of antibody D7324 as a solid-phase capture antibody.

Epitope exposure on oligomeric gp120-gp41 complexes. To assess which regions of gp120 are accessible for antibody binding on the surface of multimeric gp120-gp41 complexes, we analyzed the binding of MAbs to HIV-1 HxB2-infected cells by immunofluorescence and flow cytometry (Fig. 3 and Table 5). To eliminate the possibility that temperature might influence MAb binding, in preliminary experiments test incubations were performed at three temperatures: 4, 22, and 37°C. No substantial changes in MAb reactivity were seen at the different temperatures, so staining was routinely carried out at 4°C. All MAbs were initially tested at one concentration (25 μ g/ml); those MAbs giving a positive signal were retested by titration to confirm the specificity of their reactivity.

Fewer regions of the functional folded gp120 molecule were available than with the monomeric form (Fig. 3 and Table 5). Of MAbs binding within the C1 domain, 7E2/4 and M85

reactive with the extreme N terminus (Table 1) showed weak reactivity with oligomeric gp120 (Table 5). Only one other C1 MAb, 5B3, directed against peptide 91-100 (Table 1) (47), bound significantly to the gp120 oligomer. Antibodies directed against the V1 domain were not available for testing in this assay. There was no reactivity of MAb RSD-33 or 6C4/5 to the N-terminal flank of the V2 loop, but neutralizing MAbs, such as G3-136 and BAT-085, directed against the crown of the V2 loop bound with intermediate avidity to oligomeric gp120 (Table 5). The C2 region was not exposed; none of the MAbs listed in Table 2 exhibited significant binding to the cell surface. The central section of the V3 loop is readily accessible for MAb binding on oligomeric gp120 (Table 5), but the base of the loop is not (62). This pattern of V3 loop MAb reactivity with oligomeric gp120 mirrors that observed with the monomer. MAbs directed against the C3 and V4 regions (Table 3) failed to bind to oligomeric gp120, but there was weak but significant binding of MAbs to the C4 region (Table 5). Several of these C4 MAbs bind more avidly to oligomeric gp120 from the Hx10 clone than they do to HxB2 gp120 (62). The V5 and C5 domains were not exposed on oligomeric gp120; none of the MAbs listed in Table 4 stained the cell surface significantly.

DISCUSSION

Our principal conclusion is that only a small proportion of the constituent amino acids of gp120 are readily accessible to antibodies that bind continuous determinants when the molecule is properly folded. By using, where possible, multiple MAbs directed against the same region, we have tried to reduce the chances of obtaining misleading results, because it has been well documented that a short, continuous epitope may adopt very different conformations in a peptide and in situ on a protein (26, 76). Our studies with MAbs directed against the 91-100 region in C1 demonstrate this point: several MAbs

TABLE 4. MAb epitope exposure in the gp120 V5 and C5 domains

MAb ^a	Reference ^b	MAb-peptide reactivity		HxB2 gp120 mutant ^c with impaired binding to MAb		Relative affinity ^d of MAb for:	
		Peptide	OD ₄₉₂	Native gp120 ^e	Denatured gp120 ^f	Denatured gp120 ^g	ΔV1/2/3 ^h
M91	72	451-470	1.394	470P/L	470P/L	24	1.0
		461-480	1.264	477D/V			
CRA1 (ADP 323)		451-470	0.315	470P/L	470P/L	24 (33)	0.2
		461-480	1.936	470P/G 475M/S 477D/V 485K/V	475M/S		
9301		471-490	1.339			19 (30)	2.6
1C1		471-490	1.657	485K/V		15 (100)	0.5
221 (ADP 301)		471-490	1.141	477D/V		12 (100)	
660-178		471-490	1.671	None	485K/V	>100	
8C6/1		471-490	1.715		485K/V	>30	
5F4/1		471-490	1.554		485K/V	>10	
3F5		471-490	1.139			100	
W2		472-491	0.560		475M/S 485K/V	30	
RV110026		481-500	1.706			(15)	
		491-510	1.765				
110.1	21	481-500	0.242			(0.7)	
		491-510	1.821				

^a The immunogens for all the MAbs were envelope glycoprotein preparations that contain predominantly correctly folded molecules.

^b Citation for original description of MAb; donors of unpublished MAbs are listed in Materials and Methods.

^c Mutants highlighted by bold type show very weak or no binding.

^d Measured in fold increase in binding.

^e The test panel also contained the 470P/G and 477D/V mutants, except for MAb M91 when 470P/G was not tested.

^f The 491I/F and 493P/K mutants were not tested.

^g SDS-DTT-denatured gp120 compared with native gp120. Values in parentheses are derived from the assay using polyclonal serum R1/87 instead of the C5 antibody D7324 as capture reagent.

^h $\Delta V1/2/3$ mutant compared with wt HxB2 gp120. A blank entry indicates that the MAb was not tested.

bind equally well to native and denatured gp120, and other MAbs recognize preferentially denatured gp120, yet all are strongly reactive with the same peptides (Table 1). The influence of surrounding amino acids on the structure of an epitope on a protein's surface can be very important (76).

Within the limitations of our techniques, our data show that on the recombinant, monomeric BH10 or HxB2 molecules, the major exposed regions are the amino terminus from approximately amino acids 31 to 105 (C1); the central regions of the V1, V2, and V3 loops; part of the C4 region; and the extreme carboxy terminus (C5). These findings are summarized in Fig. 3 and 4. However, on the oligomeric envelope glycoprotein complex, only the V2 and V3 variable loops and a segment of C4 are well exposed as continuous epitopes, with the proviso that definitive information on the exposure of V1 is not yet available. Presumably, the regions in C1 and C5 that are antibody accessible on the gp120 monomer are occluded either by gp41 or by other gp120 molecules, or they adopt different

conformations, when the oligomeric complex is formed; indeed, mutagenesis studies indicate that the gp120 regions most crucial for interaction with gp41 lie within the C1 and C5 regions (17). Furthermore, the C4 section from amino acids 430 to 438 is less well exposed on oligomeric gp120 than on the monomer (62). This inaccessibility on the oligomer of parts of the C1 and C4 regions probably explains why certain MAbs to these regions can effectively block the interaction of monomeric, recombinant gp120 with CD4 yet are relatively poor at neutralizing HIV-1 (9, 31, 47, 62, 68). Other C4 MAbs are

TABLE 5. Reactivities of MAbs with oligomeric gp120-gp41 complexes

Epitope cluster	MAb	Reactivity ^a
C1	7E2/4	6.15
	M85	4.25
	5B3	19.01
V2	G3-136	21.44
	BAT085	9.38
V3	BAT123	49.00
	110.5	69.49
	9284	57.66
C4	G3-42	6.88
	G3-299	7.12
	G3-519	7.41

^a Each value represents the mean fluorescence intensity minus the background staining with MAb at 25 μ g/ml in one representative experiment.

FIG. 3. Reactivities of MAbs with monomeric and oligomeric HxB2 gp120 glycoproteins. MAb binding to native, monomeric gp120 by ELISA (top) or to the functional gp120-gp41 oligomeric complex by flow cytometry (bottom) is represented in pictorial form. The strength of the MAb reactivity is represented by the degree of shading; the most accessible regions are white, and the least accessible regions are black.

FIG. 4. Summary of MAb epitope exposure on monomeric gp120 glycoproteins. The schematic representation of gp120 is based on that of Leonard et al. (27) and is used with permission of the author. Areas not highlighted by blocks are those for which we lack information. The remaining areas are shaded according to their relative exposure on native, monomeric recombinant BH10 gp120: the lighter the shading, the greater the exposure. Boundaries between sections of different exposure are approximate. When MAb epitopes can be determined with greater precision, the length of shaded segments may alter, probably by decreasing. The dotted lines around amino acids 423 to 430 indicate an exposed area of uncertain extent.

clearly potent neutralizing antibodies (48); the precise geometries of the various C4 epitopes presumably influence the neutralization efficiencies of MAbs interactive with them. Antibodies which block the binding of monomeric gp120 to CD4 are also present at high titers in HIV-1-positive sera (7, 37, 41, 63). While a significant fraction of these antibodies may recognize discontinuous epitopes that are accessible as neutralization targets on the virus (37), others will be inert under in vivo conditions if they are unable to bind to oligomeric gp120. Thus, in vitro assays for gp120 CD4-blocking antibodies using recombinant, monomeric glycoproteins may correlate only roughly with virus neutralization assays (62).

In terms of gp120 structure, our results suggest that most of the conserved regions might lie within the core of the molecule, presumably necessary for its structure and function. This is consistent with the hydrophobic nature of most of the

conserved gp120 regions. That many of these regions are not immunogenic on the native gp120 glycoprotein is attested to by the observation that some of the MAbs recognizing these regions were not raised with the properly folded gp120 protein. However, many MAbs with similar properties were raised against gp120 or gp160 preparations containing predominantly correctly folded molecules, although we cannot know the shape of the particular molecule that was immunogenic for any given MAb. It is also notable that most of the continuous regions of gp120 for which we currently lack MAbs lie within the conserved regions. These negative results further support the conclusion that few of the continuous, conserved regions on native, monomeric gp120 and even fewer on the oligomer are antibody accessible: if these regions were well-exposed, we would expect them to be immunogenic.

The gp120 regions that are best exposed on the oligomer are

the V2 and V3 loops, known sites for neutralizing antibodies. The possibility that the V1 loop of HIV-1 gp120 also represents a neutralization epitope (54) suggests that it may also be exposed on oligomeric gp120. MAbs directed against the V4 and V5 loops are very rare and do not bind well to the native monomeric or oligomeric gp120 molecule. Their variability suggests that the V4 and V5 domains could be subject to immune selection pressure, as occurs with the V2 and V3 loops (50, 80), although this need not indicate direct interaction with antibodies or surface accessibility (8). Thus, a model of HIV-1 gp120 consistent with the experimental data would have the variable loops predominantly on the exterior and the conserved regions mostly in the interior. The inaccessibility of the continuous, conserved domains on virion-associated gp120 may be an important factor contributing to their conservation; they are not subject to immune selection. However, the variable loops do not appear merely to shield the conserved domains of gp120 from antibodies; deletion of the V1, V2, and V3 loops did not cause any of the continuous, conserved regions to become significantly more accessible to antibodies, and the increase in antibody binding to the $\Delta V1/2/3$ molecule on its denaturation was always similar to that seen with the wt gp120 proteins (data not shown).

While few continuous, conserved regions appear to be exposed on the surface of gp120, smaller segments of the conserved regions must be exposed, perhaps only transiently, for CD4 binding. Since continuous antibody epitopes typically consist of at least four to six amino acids (26), antibody mapping is a very low resolution technique unsuited to the delineation of small exposed segments of gp120. Amino acids critical for the interaction of gp120 with CD4 have been localized to the C2, C3, and C4 domains (53). Our present view of the CD4-binding site is that it is a complex structure comprising amino acids widely separated in the gp120 primary sequence but brought into proximity in the folded molecule. The site may be a canyon with walls made from different gp120 domains. Thus, while larger continuous stretches of C2, C3, and C4 amino acids are not surface accessible, individual amino acids from these regions could contribute to a discontinuous CD4-binding structure. Likewise, portions of poorly accessible, conserved regions could contribute residues to discontinuous antibody epitopes. For example, the C2 region around Thr-257 is important for CD4 binding (53) and for the epitopes of some of the neutralizing MAbs directed against the CD4-binding site (69, 71). This C2 region is well represented in MAb panels raised against monomeric gp120, yet none of the MAbs reactive with linear C2 determinants binds preferentially to native gp120 and none is neutralizing. Presumably some elements of the C2 region proximal to Thr-257 are exposed on at least a fraction of the gp120 molecules used as the immunogen. Since antibodies to discontinuous epitopes affected by substitutions at Thr-257 neutralize HIV-1 (69, 71), these elements may be exposed at some period during viral entry. Alternatively, substitutions in this C2 region and indeed in other conserved domains might affect discontinuous antibody epitopes indirectly through alteration of gp120 folding; there is no formal proof that, for example, Thr-257 is a contact amino acid for either CD4 or neutralizing antibodies, although it might well be so. What our studies show is that the continuous segment from residues 252 to 261 is poorly exposed in the context of the native gp120 glycoprotein; this is also the case for most other conserved regions of gp120.

The extensive glycosylation of the HIV-1 gp120 glycoprotein may contribute to the inaccessibility of the conserved gp120 regions to antibodies. There are up to 30 N-linked glycosylation sites on HIV-1 gp120 (27, 46) and perhaps also several

O-linked sites (16). The size of these carbohydrate units (approximately 2,000 Da) is not always appreciated. For comparison, the entire V3 domain of gp120 has a peptide molecular mass of about 4,000 Da. There is some evidence that carbohydrates may shield antibody epitopes (3, 11, 52), presumably by virtue of their bulk, although this is not certain. It is notable that glycosylation sites with complex carbohydrates (27) tend to be in regions of gp120 we assess to be relatively well exposed on the gp120 surface. The modifying enzymes that create the complex carbohydrate chains act on gp120 once it has folded (13) and may not have access to the unmodified, high-mannose glycosylation sites closer to the interior of the protein. Carbohydrates on the latter type of site may have an important role in gp120 folding and the preservation of its structure.

We know of very few antibodies to gp120 (or for that matter to gp41) that can be demonstrated to bind strongly to an intact gp120-gp41 oligomeric complex that are not neutralizing. Two candidates for such MAbs are M90 (12) and 5B3 (Table 5) (47), but they are rare. We suggest that HIV-1 has evolved a glycoprotein structure that minimizes the antibody-accessible surface both by preserving key structural elements in the interior of the molecule and by covering the structure with poorly immunogenic carbohydrates. Furthermore, those well-exposed regions, notably the V2 and V3 loops, can mutate to destroy antibody epitopes without loss of function. Together, these devices offer a considerable selective advantage to HIV-1 in body compartments rich in anti-gp120 antibodies.

Finally, it is not unreasonable to want to raise antibodies against novel epitopes in the conserved regions of gp120 (57), but if these antibodies fail to bind the gp120-gp41 oligomer in situ, they will not neutralize it. Our experience with antibodies raised against a denatured form of gp160 (1, 40) is that they recognize denatured gp120 well but bind poorly to native, monomeric gp120 and not at all to the oligomer. These antibodies do not neutralize HIV-1 (1). However, these properties are also shared by most MAbs raised against native forms of recombinant or virus-derived gp120 or gp160, which constitute the majority of the MAbs in the panel we have used in this study. The high prevalence of MAbs to poorly exposed continuous epitopes in (especially) the amino-terminal halves of C1 and C2 and short segments of C3 and C5 is clear from our tabulated data. We have used more than 75 such MAbs in the present study, yet we know of only about 7 mouse or rat MAbs against gp120 that recognize discontinuous epitopes, in that they are peptide unreactive and their binding to gp120 is disrupted by mutant amino acids scattered throughout gp120. In marked contrast, most human MAbs raised in vivo against virion gp120-gp41 recognize discontinuous epitopes; with the exception of MAbs directed against the V3 loop, only 3 of more than 50 human MAbs directed against gp120 of which we are aware recognize gp120 continuous epitopes, each binding to the extreme C terminus. Furthermore, most polyclonal anti-gp120 antibodies in HIV-1-positive sera recognize discontinuous epitopes (37). Even allowing for differences in screening techniques, this difference is striking. The high proportion of murine MAbs to monomeric gp120 or gp160 that are directed against conserved, continuous, but poorly accessible epitopes may reflect, in part, the presence of misfolded, perhaps abnormally immunogenic, molecules (20). This may be exacerbated in animal immunizations by the use of adjuvants such as Freund's which could contribute to gp120 unfolding.

Clearly, a major challenge for HIV-1 vaccine development is a determination of means to elicit neutralizing antibodies recognizing more-conserved elements of the envelope glyco-

proteins. All of the conserved gp120 neutralization targets defined to date are discontinuous and either overlap the CD4-binding site (69, 71) or are induced in their exposure by CD4 binding (70). These antibodies are rarely induced following immunization even with correctly folded recombinant gp120-gp160 glycoproteins, although several have now been identified (9, 18, 43, 47, 48, 12). The apparent masking of some of the discontinuous epitopes overlapping the CD4-binding site on both monomeric and oligomeric envelope glycoproteins by the V1/V2 and V3 variable loops (77) could contribute to this poor immunogenicity. Additional studies of gp120 structure may uncover other viral mechanisms designed to minimize the immunogenicity of the HIV-1 envelope glycoproteins and may suggest approaches to overcome such mechanisms.

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