Characterization of the Multiple Conformational States of Free Monomeric and Trimeric Human Immunodeficiency Virus Envelope Glycoproteins after Fixation by Cross-Linker

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The human immunodeficiency virus type 1 (HIV-1) gp120 exterior and gp41 transmembrane envelope glycoproteins assemble into trimers on the virus surface that represent potential targets for antibodies. Potent neutralizing antibodies bind the monomeric gp120 glycoprotein with small changes in entropy, whereas unusually large decreases in entropy accompany gp120 binding by soluble CD4 and less potent neutralizing antibodies. The high degree of conformational flexibility in the free gp120 molecule implied by these observations has been suggested to contribute to masking the trimer from antibodies that recognize the gp120 receptor-binding regions. Here we use cross-linking and recognition by antibodies to investigate the conformational states of gp120 monomers and soluble and cell surface forms of the trimeric HIV-1 envelope glycoproteins. The fraction of monomeric and trimeric envelope glycoproteins able to be recognized after fixation was inversely related to the entropic changes associated with ligand binding. In addition, fixation apparently limited the access of antibodies to the V3 loop and gp41-interactive surface of gp120 only in the context of trimeric envelope glycoproteins. The results support a model in which the unliganded monomeric and trimeric HIV-1 envelope glycoproteins sample several different conformations. Depletion of particular fixed conformations by antibodies allowed characterization of the relationships among the conformational states. Potent neutralizing antibodies recognize the greatest number of conformations and therefore can bind the virion envelope glycoproteins more rapidly and completely than weakly neutralizing antibodies. Thus, the conformational flexibility of the HIV-1 envelope glycoproteins creates thermodynamic and kinetic barriers to neutralization by antibodies directed against the receptor-binding regions of gp120.

Human immunodeficiency virus type 1 (HIV-1) is the etiologic agent of most cases of AIDS (3, 23). The epidemic of HIV-1 infections continues to expand globally, with more than 40 million humans currently infected by the virus (27a). Modalities to prevent transmission of HIV-1 are urgently needed to curb the AIDS epidemic. The early events in HIV-1 infection, which occur before the formation of the provirus, serve as attractive targets in the development of prophylactic approaches, including vaccines.

HIV-1 entry, which occurs in water-soluble compartments readily accessible to drugs and involves well-defined viral and host molecules, has generated much interest as a target for intervention. HIV-1 entry into the host cell is mediated by the viral envelope glycoproteins, which are derived by proteolytic cleavage of a trimeric, glycosylated gp160 envelope glycoprotein precursor (2, 46). The resulting mature envelope glycoproteins, gp120 and gp41, constitute a trimeric complex on the virion surface that is anchored by the membrane-spanning segments of the gp41 transmembrane envelope glycoproteins (6, 7, 16, 17, 37, 42, 58). The gp120 exterior envelope glycoprotein is retained on the trimer via labile, noncovalent interactions with the gp41 ectodomain (26). The gp120 glycoprotein is the most exposed element on the trimer and binds the initial

receptor, CD4 (11, 28). CD4 binding triggers conformational changes in gp120 that promote its interaction with one of the chemokine receptors, CCR5 or CXCR4 (1, 10, 13–15, 19, 54, 61). CD4 binding also induces conformational changes within the assembled HIV-1 envelope glycoprotein trimer that result in the exposure of a helical heptad repeat (HR1) segment of the gp41 ectodomain (22, 25, 29, 50). Eventually, the conformational transition of the gp41 ectodomain into a six-helix bundle composed of the HR1 and HR2 heptad repeat regions is thought to provide the energy needed to fuse the viral and target cell membranes (7, 37, 58).

The binding of an antibody molecule to the HIV-1 envelope glycoprotein complex results in neutralization of the function of the bound trimer (69). Thus, the ability of HIV-1 to establish persistent infections in human hosts requires envelope glycoprotein characteristics that minimize the elicitation and efficacy of neutralizing antibodies (4, 64). Indeed, during natural HIV-1 infection, antibodies that potently neutralize primary clinical HIV-1 isolates are only rarely elicited. Several features of the HIV-1 gp120 envelope glycoprotein that are important in evasion of the host immune response include heavy glycosylation, sequence variability, and conformational masking of conserved epitopes involved in receptor binding (30, 38, 57, 64). The latter property has been deduced from thermodynamic studies that suggest that monomeric gp120 experiences unusually large decreases in entropy upon binding CD4 and many weakly neutralizing antibodies (30, 41). These

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ligands are hypothesized to fix the gp120 glycoprotein, which is proposed to be conformationally flexible in the free, unliganded state, into a single conformation, thus accounting for the observed decreases in entropy. By contrast, the rare potent neutralizing antibodies bind gp120 with small changes in entropy (30). Although these thermodynamic studies were carried out with monomeric gp120, the observed correlation between the entropic change associated with antibody binding and neutralization potency implies relevance to the functional HIV-1 envelope glycoprotein trimer as well (30). Presumably, the large, thermodynamically unfavorable changes that occur in the context of the envelope glycoprotein trimer minimize effective antibody binding to the virus.

Full-length HIV-1 gp120 has eluded structural analysis. However, deletion of the large V1, V2, and V3 variable loops and the N and C termini of the gp120 glycoproteins of HIV-1 and simian immunodeficiency virus (SIV) results in "gp120 core" proteins, which have been crystallized (8, 9, 31–33). High-resolution X-ray crystal structures of HIV-1 gp120 cores complexed with soluble CD4 and a CD4-induced (CD4i) neutralizing antibody have been solved (32, 33). The gp120 core consists of an inner, gp41-interactive domain, a heavily glycosylated outer domain exposed on the trimer surface, and a conformationally labile bridging sheet thought to cooperate with the V3 variable loop in binding the chemokine receptor (33, 44, 45). All three core domains contribute to the formation of the binding site for CD4 (32, 33, 63, 64). When CD4 and most antibodies directed against the receptor-binding surface of gp120 bind to the HIV-1 gp120 core, large decreases in entropy occur (30, 41). Thus, the unliganded HIV-1 gp120 core exhibits a high degree of disorder (41).

Given the evidence for the high entropy of the HIV-1 gp120 glycoprotein, it was surprising that the SIV gp120 core was able to be crystallized in the unliganded state (8, 9). Although these crystals presented many problems in analysis (8), a low-resolution structure was solved (9). In the unliganded SIV core, major differences exist in the inner domain and bridging sheet compared with these elements in the CD4-bound HIV-1 gp120 core. By contrast, the outer domains of the SIV and HIV-1 gp120 cores are similar in structure. Chen and colleagues (9) favor a model in which the SIV gp120 core structure represents the major or sole unliganded conformation on the SIV or HIV-1 envelope glycoprotein trimer.

An understanding of the different conformational states available to the HIV-1 envelope glycoproteins is critical to rational attempts to design inhibitors and vaccines. The unliganded HIV-1 envelope glycoprotein trimer is the major, and perhaps the only, viable target for neutralizing antibodies; steric factors limit the access of antibody molecules to conserved, transitional structures exposed on the surface of the CD4-bound HIV-1 envelope glycoproteins (35). Effective low-molecular-weight inhibitors may need to recognize the unliganded envelope glycoproteins as well. Therefore, we wished to address whether the unliganded HIV-1 envelope glycoproteins sample different conformational states. The preservation of a limited number of epitopes on the HIV-1 envelope glycoproteins after formalin fixation has been previously examined (47a). Here, we use the efficient cross-linking agent glutaraldehyde (GA) to fix the conformations of monomeric and trimeric HIV-1 envelope glycoproteins. We probe these con-

formational states with a panel of gp120-directed ligands, including potent and weak neutralizing antibodies, and relate the results to thermodynamic binding data. We document the existence of distinct conformations of the HIV-1 gp120 glycoprotein in both monomeric and trimeric contexts, characterize the representation of these conformational states in the unliganded envelope glycoproteins, and investigate the consequences of gp120 conformational flexibility on the kinetics of ligand binding.

MATERIALS AND METHODS

Envelope glycoprotein constructs. All of the soluble envelope glycoproteins used in this study were derived from the YU2 primary R5 HIV-1 isolate. The sgp140(–) glycoprotein consists of the complete gp120 and gp41 ectodomain with alterations in the gp120/gp41 cleavage site (arginines at amino acid positions 508 and 511 changed to serines). The sgp140(–/GCN4) glycoprotein contains the sgp140(–) sequence with a GCN4 trimerization motif appended to the carboxyl terminus of the gp41 ectodomain (68, 70).

Expression and cross-linking of envelope glycoproteins. The envelope glycoproteins were either transiently expressed in 293T cells or stably expressed in Chinese hamster ovary (CHO) cells. For transient expression, 293T cells were transfected with *env*-expressing plasmids by using Lipofectamine PLUS transfection reagent and following the manufacturer's protocol (Invitrogen). Twenty-four hours after the start of transfection, the expressed envelope glycoproteins were radiolabeled with [³⁵S]methionine for 36 h prior to harvesting. The envelope glycoproteins expressed stably in CHO cell lines were also radiolabeled with [³⁵S]methionine for 36 h prior to harvesting. The harvested culture supernatants were centrifuged at low speed to clear the cell debris, and the envelope glycoproteins were cross-linked as previously described (71). Briefly, 100 μ l of supernatants were diluted with 300 μ l of phosphate-buffered-saline (PBS) containing 1 mM EDTA and incubated with various concentrations of GA (Sigma) at room temperature for 5 min, followed by addition of glycine to a final concentration that was 10-fold higher than that of GA to quench the unreacted GA.

Immunoprecipitation of envelope glycoproteins. One-hundred microliters of cell supernatants containing radiolabeled envelope glycoproteins were mixed with 900 μ l PBS and incubated with 1 μ l of a mixture of pooled sera (PS) from HIV-1-infected individuals, with 1 μ g of individual monoclonal antibodies that recognize different envelope glycoprotein epitopes, or with 1 μ g of the CD4-immunoglobulin (CD4-Ig) fusion protein. The antibody-glycoprotein complex was precipitated by protein A-Sepharose beads (Amersham Biosciences) and washed twice with PBS containing 0.2% Tween 20 before analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The radiolabeled envelope glycoprotein bands on the SDS-PAGE gels were quantified with a STORM system and a PhosphorImager screen (Molecular Dynamics).

For the depletion of specific monoclonal antibody-reactive envelope glycoproteins, the supernatants containing radiolabeled envelope glycoproteins were either left untreated or cross-linked and were then incubated with the monoclonal antibody and protein A-Sepharose beads. The envelope glycoproteins reactive with the monoclonal antibody were precipitated, and the remaining supernatants were incubated with fresh monoclonal antibody and protein A-Sepharose beads. After precipitation, the supernatants were subjected to two additional precipitations with the same monoclonal antibody. Pilot experiments confirmed that the depletion of envelope glycoproteins capable of being precipitated by the monoclonal antibody was complete. The envelope glycoproteins left in the supernatants after the depletion were then divided and used for precipitation by PS or other monoclonal antibodies. The precipitates were washed and the envelope glycoproteins subjected to SDS-PAGE as described above.

For the kinetic study of antibody recognition of soluble HIV-1 envelope glycoproteins, the different antibodies were first coupled to protein A-Sepharose beads by incubating them together at room temperature for 2 h. After being washed twice with PBS, the antibody-coupled protein A beads were incubated with mixed supernatants containing radiolabeled gp120 and sgp140(–/GCN4) envelope glycoproteins at 4°C for various time periods. The precipitates were washed, and the envelope glycoproteins were subjected to SDS-PAGE as described above.

Fluorescence-activated cell sorter (FACS) analysis of HIV-1-infected cells. Jurkat cells were infected with wild-type HIV-1 NL4-3, an X4 strain. PM1 cells were infected with HIV-1 NL4-3(YU2), an NL4-3 variant in which the NL4-3 *env* gene is replaced with that derived from the R5 HIV-1 YU2 strain. The infected

cells were propagated for 1 to 3 weeks and were either left untreated or cross-linked with 5 mM GA before staining with antibodies.

For GA cross-linking, the cells were pelleted by low-speed centrifugation and resuspended in PBS containing 1 mM EDTA. GA was added to a final concentration of 5 mM. The cell suspension was incubated at room temperature for 5 min before addition of 2 volumes of PBS containing 50 mM glycine. After a further 10-min incubation at room temperature, the cells were pelleted and washed with PBS containing 0.5% bovine serum albumin (BSA) and 0.02% azide.

For antibody staining, the cells were pelleted and incubated in 100 μ l of PBS–0.5% BSA–0.02% azide containing 1 μ l of PS or 1 μ g of different monoclonal antibodies or the CD4-Ig fusion protein at 4°C for 2 to 3 h. After being washed once with PBS–0.5% BSA–0.02% azide, the antibodies bound on the cell surface were stained with 1 μ l of fluorescein isothiocyanate (FITC)-conjugated anti-human Ig antibody (Pierce) in 100 μ l of PBS–0.5% BSA–0.02% azide at 4°C for 1 h. The cells were washed twice with PBS–0.5% BSA–0.02% azide before being subjected to FACS analysis.

For the kinetic analysis of antibody and CD4-Ig binding to cell surface HIV-1 envelope glycoproteins, the HIV-1 NL4-3(YU2)-infected PM1 cells were pelleted and incubated in PBS–0.5% BSA–0.02% azide containing 10 μ g/ml of different monoclonal antibodies or the CD4-Ig fusion protein at room temperature for various time periods. After being washed twice with PBS–0.5% BSA–0.02% azide, the antibodies bound on the cell surface were stained with 1 μ l of FITC-conjugated anti-human Ig antibody in 100 μ l of PBS–0.5% BSA–0.02% azide at 4°C for 1 h. The cells were washed twice with PBS–0.5% BSA–0.02% azide before being subjected to FACS analysis.

RESULTS

Effects of cross-linking on the antigenicity of soluble HIV-1 envelope glycoproteins. We wished to investigate the conformation(s) of the unliganded HIV-1 gp120 envelope glycoprotein, both as a monomer and in the context of a trimer. The HIV-1 envelope glycoproteins used in this study were derived from the YU2 strain and include gp120, cleavage-defective soluble gp140 [sgp140(–)], and cleavage-defective soluble gp140 stabilized by a heterologous GCN4 trimerization motif [sgp140(–/GCN4)]. The majority of the sgp140(–) glycoprotein forms dimers and trimers, whereas the sgp140(–/GCN4) glycoprotein forms stable trimers (71). The radiolabeled soluble envelope glycoproteins were cross-linked with 5 mM or 8 mM GA. GA cross-links lysine residues and thereby fixes the conformation of the protein component of the envelope glycoproteins (12, 34, 60). We showed previously that GA at concentrations above 5 mM fully cross-linked soluble HIV-1 envelope glycoprotein oligomers (71). Therefore, we chose 5 mM and 8 mM GA to compare the effects of cross-linking the HIV-1 envelope glycoproteins at and beyond saturating concentrations, respectively. The untreated and cross-linked envelope glycoproteins were immunoprecipitated either with PS from HIV-1-infected individuals or with a panel of monoclonal antibodies that recognize different conformation-dependent envelope glycoprotein epitopes; the precipitated proteins were then analyzed by SDS-PAGE (Fig. 1) and quantified with a PhosphorImager screen (Fig. 2). Antibody recognition of the dimeric and trimeric forms of sgp140(–) was similar; therefore, the quantification of sgp140(–) after cross-linking was applied only to the trimers. The PS are known to recognize both native and denatured forms of the HIV-1 envelope glycoproteins (2, 39, 46, 65); therefore, the amount of envelope glycoprotein precipitated by the PS serves as a measurement of the total precipitable envelope glycoprotein in the supernatants. For untreated and cross-linked samples, the amount of envelope glycoproteins precipitated by individual monoclonal

FIG. 1. Effects of cross-linking on the antigenicity of soluble envelope glycoproteins. (A) The radiolabeled gp120, sgp140(–), and sgp140(–/GCN4) glycoproteins from the YU2 strain of HIV-1 were either left untreated or cross-linked with 5 mM or 8 mM GA before being immunoprecipitated by PS or different monoclonal antibodies. The precipitates were subjected to SDS-PAGE and autoradiography. (B) The radiolabeled gp120 and sgp140(–/GCN4) glycoproteins were either left untreated or treated with 5 mM or 8 mM GA before being precipitated by PS or CD4-Ig. Precipitates were analyzed by SDS-PAGE.

FIG. 2. Relative recognition of cross-linked and untreated HIV-1 envelope glycoproteins by ligands. (A) The radiolabeled envelope glycoproteins precipitated in experiments similar to those shown in Fig. 1 were quantified with a STORM system and a PhosphorImager screen. In the case of cross-linked sgp140(-), the quantification was applied only to the trimer bands. The amounts of envelope glycoproteins precipitated by individual monoclonal antibodies were compared to that precipitated by PS with the same treatment. In panels B and C, the amounts of envelope glycoproteins precipitated by CD4-Ig and the 2G12 antibody, respectively, compared to that precipitated by PS with the same treatment are shown

antibodies was compared to that precipitated by PS. The results obtained after cross-linking with 5 mM and 8 mM GA were similar; these results, and those shown below (Fig. 2D), indicate that saturation is achieved at GA concentrations near or beyond 8 mM. The effects of cross-linking on antibody binding varied with the different monoclonal antibodies and different envelope glycoprotein constructs (Fig. 1A and 2A). The results are summarized below:

(i) CD4-binding site (CD4BS) antibodies recognize HIV-1 gp120 epitopes that overlap the binding site for CD4 but are thought to interact with conformations of gp120 that are distinct from that recognized by CD4 (63, 67). CD4BS antibodies include both potent (e.g., IgG1b12, herein designated b12) and less potent neutralizing agents (e.g., F105 and 15e).

The recognition of the monomeric gp120 glycoprotein by the b12 antibody was less efficient than recognition of the trimers by this antibody. The interactions of the b12 antibody with the monomeric and trimeric envelope glycoproteins were minimally decreased by GA treatment (Fig. 1A and 2A), indicating that the epitope for the b12 antibody was efficiently maintained on the cross-linked envelope glycoproteins.

We also studied two other CD4BS antibodies, F105 and 15e, that exhibit only limited potency in neutralizing primary HIV-1 isolates. The F105 and 15e antibodies precipitated the cross-linked envelope glycoproteins between 20 and 35% as efficiently as they precipitated the corresponding untreated HIV-1 envelope glycoproteins (Fig. 1A and 2A).

(ii) 2G12 is a potentially neutralizing and broadly reactive antibody that recognizes a cluster of oligomannose residues added posttranslationally to the gp120 outer domain (47, 49, 55). The interactions of the 2G12 antibody with the envelope glycoproteins, both monomers and trimers, were minimally affected by GA cross-linking (Fig. 1A and 2C).

(iii) CD4i antibodies recognize gp120 epitopes that overlap the chemokine receptor-binding site and that are formed and exposed after CD4 binding (45, 53, 66). CD4i antibodies exhibit low neutralizing activity against clinical HIV-1 isolates. Cross-linking the HIV-1 envelope glycoproteins dramatically reduced recognition by two CD4i antibodies, 17b and E51 (Fig. 1A and 2A).

(iv) C11 and 2/11c are two nonneutralizing antibodies that bind to conformation-dependent gp120 epitopes that are involved in the interaction with gp41 (36, 40, 54, 62). The C11 and 2/11c epitopes are thought to be occluded on the functional HIV-1 envelope glycoprotein trimer (63). Even in the absence of cross-linking, the binding of C11 or 2/11c with the sgp140(–) or sgp140(–/GCN4) trimer was decreased compared with gp120 monomer binding. Cross-linking the sgp140(–) or sgp140(–/GCN4) trimer further reduced the

interaction with C11 or 2/11c to undetectable or low levels, respectively (Fig. 1A and 2A).

(v) The 39F antibody interacts with the V3 loop of gp120 (30), which is a region critical for chemokine receptor binding (44, 45). Because of epitope variability, antibodies against the gp120 V3 loop usually, although not always, have a narrow breadth of reactivity (4). Nonetheless, V3-directed antibodies can often potently neutralize viruses that are recognized. The recognition of monomeric and trimeric HIV-1 envelope glycoproteins by the 39F antibody was only minimally affected by cross-linking (Fig. 1A and 2A).

(vi) A soluble form of CD4, CD4-Ig (5), recognized the monomeric and trimeric HIV-1 envelope glycoproteins inefficiently after cross-linking (Fig. 1B and 2B).

To verify that the GA concentrations used in the above experiments were saturating, the ability of several antibodies to precipitate the HIV-1 gp120 glycoprotein after treatment with increasing GA concentrations was examined. The recognition of cross-linked gp120, relative to that of untreated gp120, for each of the antibodies was similar at GA concentrations at and beyond 8 mM (Fig. 2D). Thus, 8 mM GA is sufficient to cross-link the HIV-1 gp120 glycoprotein completely.

The effect of antibody concentration on the precipitation of HIV-1 gp120 cross-linked with 8 mM GA was studied (Fig. 2E and data not shown). The maximum amount of cross-linked gp120 able to be precipitated differed for each antibody. Estimation of the antibody concentration required to achieve 50% of the maximal binding suggested that the antibodies exhibit similar affinities for the fraction of gp120 glycoproteins that can still be recognized after cross-linking. Thus, each antibody efficiently recognizes a fraction of the cross-linked gp120 population; this fraction is a property of the particular antibody.

With the exception of 2G12, which binds HIV-1 YU2 gp120 with reduced affinity compared with the other ligands, the antibodies described above bind HIV-1 gp120 with similar affinities. However, a wide range of entropic changes is associated with the binding of these ligands to gp120 (30) (Fig. 3A). We wished to use the recognition of GA-treated envelope glycoproteins by these ligands to test the hypothesis that multiple conformational states are sampled by the unliganded HIV-1 envelope glycoproteins (the multistate model). Theoretically, the occupancy of each conformational state at equilibrium will be predicted by a Boltzmann distribution (24, 56), according to the free energy associated with each conformation (Fig. 3B). If an excess of a ligand that is associated with a small entropy change ($-T\Delta S$) upon gp120 binding (e.g., 39F, 2G12, or b12 in Fig. 3A) is added to the system, the multistate model predicts that little or no change in the distribution of gp120 molecules among the different conformational states will oc-

for each envelope glycoprotein. V3 indicates recognition of the V3 loop of gp120. CD4BS indicates the antibodies recognizing CD4 binding site epitopes. CD4i indicates the antibodies recognizing CD4i epitopes. Non-Neut. indicates antibodies with no neutralizing activity. The values reported are representative of those obtained in three separate experiments. (D) The radiolabeled gp120 glycoprotein from the YU2 strain of HIV-1 was either left untreated or cross-linked with the indicated concentrations of GA before being immunoprecipitated by different monoclonal antibodies. The precipitates were quantified, and the amounts of GA-cross-linked gp120 precipitated by individual monoclonal antibodies were compared to that of the untreated gp120. (E) The radiolabeled gp120 glycoprotein from the YU2 strain of HIV-1 was cross-linked with 8 mM GA before being immunoprecipitated by the indicated concentrations of monoclonal antibodies. The precipitates were subjected to SDS-PAGE and quantified with a STORM system and a PhosphorImager screen.

FIG. 3. Predicted relationship between entropic change and relative recognition of fixed envelope glycoproteins. (A) The entropy changes ($-T\Delta S$) determined for the binding of the indicated ligand to HIV-1 gp120 are listed. The ratio of ligand recognition of the gp120 or sgp140(-/GCN4) glycoprotein cross-linked with 8 mM GA to that of the untreated glycoprotein was determined as described in the legend to Fig. 2. In panel B, the hypothetical conformational states of the unliganded HIV-1 envelope glycoproteins are designated A, B, and C. The occupancy of these states at equilibrium is predicted by the Boltzmann distribution (24, 56). At equilibrium, untreated envelope glycoproteins incubated either with a C-prefering ligand (red) associated with a large $-T\Delta S$ or with a nondiscriminating ligand (green) associated with a small $-T\Delta S$ will assume the conformational states shown on the left. Both ligands should bind the untreated envelope glycoproteins equivalently, given their comparable affinities. Upon fixation by

cur. By contrast, the addition of an excess of a ligand associated with large entropic changes (e.g., CD4, most CD4BS antibodies, CD4i antibodies) would be predicted to drive the population of gp120 glycoproteins into a single conformation (or a small number of conformations); according to the multistate model, this homogenization of conformations in the gp120 population accounts for the increased order (loss of entropy) in the system. Because, with the exception of 2G12, the affinities of the gp120 ligands listed in Fig. 3A are similar (30), the efficiencies with which “small $-T\Delta S$ ” or “large $-T\Delta S$ ” ligands bind gp120 will be indistinguishable at equilibrium (Fig. 3B, left side). However, by chemically cross-linking and fixing the conformations of the gp120 molecules in the system, we can reveal the existence of any distinct conformational states. After cross-linking, according to the multistate model, small $-T\Delta S$ ligands should still be able to recognize a large proportion of the fixed gp120 molecules in the population (Fig. 3B, right side). By contrast, large $-T\Delta S$ ligands would be expected to recognize a proportionately smaller fraction of the fixed gp120 molecules in the population.

We examined the relative recognition of fixed, untreated envelope glycoproteins as a function of the measured entropic change ($-T\Delta S$) associated with ligand-gp120 binding (30). For both monomeric gp120 and sgp140(-/GCN4) preparations, there was a clear inverse relationship between relative recognition and $-T\Delta S$ (Fig. 3C).

The relative recognition of the cross-linked and untreated forms of monomeric gp120 by the panel of antibodies tested is consistent with the values predicted by consideration of $-T\Delta S$ alone (Fig. 3A and C). By contrast, the relative recognition of the fixed, untreated sgp140(-) and sgp140(-/GCN4) trimers by two antibodies, 2/11c and C11, is not explained solely by the entropy change associated with binding (Fig. 3A and C and data not shown). This suggests that cross-linking of the trimers, but not the gp120 monomer, limits the access of the 2/11c and C11 antibodies to their epitopes. This interpretation is consistent with the expectation that these antibodies recognize gp120 regions that interact with gp41 in the context of the HIV-1 envelope glycoprotein trimer (40, 62).

Among the monoclonal antibodies examined in the present study, b12 and 2G12 exhibit potent and broad neutralizing activity against primary HIV-1 isolates; 39F also potently neutralizes a much more limited subset of HIV-1 isolates; F105, 15e, 17b, and E51 exhibit only limited efficiency in neutralizing primary HIV-1 isolates; C11 and 2/11c exhibit no neutralizing activity. Correlatively, the interaction of cross-linked sgp140(-) and sgp140(-/GCN4) trimers was efficient with b12, 2G12, and 39F, decreased with F105 and 15e, and minimal with C11 and 2/11c (Fig. 1A). Thus, cross-linking of the soluble envelope glycoprotein trimers preferentially maintains the binding of the

treatment with cross-linker (magenta, right side), the relative recognition (recognition of cross-linked/untreated envelope glycoprotein) of the large $-T\Delta S$ ligand is predicted by the multistate model to be less than that of the small $-T\Delta S$ ligand. (C) The relationship between the binding entropy and the relative recognition of cross-linked (8 mM GA) to untreated gp120 and sgp140(-/GCN4) envelope glycoproteins is shown for the ligands listed in panel A.

FIG. 4. Relative recognition of untreated and cross-linked HIV-1 envelope glycoproteins on infected cells. Jurkat cells infected by HIV-1 NL4-3 (top panels) or PM1 cells infected by HIV-1 NL4-3(YU2) (bottom panels) were either left untreated or treated with 5 mM GA. Cells were washed and incubated with the indicated ligand and an FITC-conjugated secondary antibody against human IgG. The cells were washed and subjected to FACS analysis (red curves). Uninfected cells were treated and analyzed in parallel (green curves).

potently neutralizing antibodies while decreasing the binding of nonneutralizing antibodies.

Effects of cross-linking on the antigenicity of cleaved, mature HIV-1 envelope glycoproteins. As soluble HIV-1 envelope glycoprotein trimers are defective for proteolytic cleavage, which can exert subtle effects on antigenicity (27, 41a, 51), we wished to investigate whether different conformational states might exist on mature HIV-1 envelope glycoprotein trimers. To this end, we studied the recognition of HIV-1 envelope glycoproteins on the surface of infected cells before and after GA treatment. The HIV-1 envelope glycoproteins on the surface of infected cells exhibit much more efficient processing than that observed in overexpressing cells (27, 41a, 51). To minimize the possible effects of cross-linking on the accessibility of the antibody to its epitope on the trimer, we compared the binding of four ligands that recognize proximal gp120 epitopes: b12, F105, 15e (all CD4BS antibodies), and CD4-Ig. PS and a V3-directed antibody, 39F, were also included in the study. All of the ligands efficiently recognized the untreated cell surface envelope glycoproteins of the NL4-3 and YU2 HIV-1 strains (Fig. 4). By contrast, after cross-linking, the cell surface envelope glycoproteins were recognized efficiently only

by the b12 antibody and PS (Fig. 4). This result is consistent with the prediction of the multistate model in which a ligand with a small $-T\Delta S$ such as b12 will recognize a larger proportion of the fixed envelope glycoproteins than ligands such as F105, 15e, or CD4-Ig with large $-T\Delta S$ values. Thus, the existence of multiple gp120 conformational states on the mature cell surface HIV-1 envelope glycoproteins is supported by these observations.

Recognition of the HIV-1 envelope glycoproteins on the infected cell surface by the 39F anti-V3 antibody was decreased after GA cross-linking (Fig. 4). This was not observed on the soluble trimers, indicating that differences exist between the soluble trimers and the native envelope glycoprotein complexes. The basis for this decrease in 39F binding requires further investigation but possibly reflects the effects of fixation on the accessibility of 39F to its V3 epitope. Adjacent structures on the trimer (for example, other variable loops) may be less flexible after cross-linking and limit the exposure of the V3 loop in this setting. Of note, the V3 loop on the R5 envelope glycoproteins appeared to be less exposed after cross-linking than the V3 loop on the X4 envelope glycoproteins. Differences in the exposure of the V3 loop on the envelope glyco-

FIG. 5. Characterization of the relationships among the conformational states of the HIV-1 envelope glycoproteins. HIV-1 YU2 monomeric gp120 (A) or the sgp140(-/GCN4) trimer (B) was either left untreated or cross-linked with 5 mM GA. The envelope glycoproteins were then sequentially precipitated (from left to right) by the ligand shown above the line. The remaining envelope glycoprotein in the supernatant was divided and precipitated with either PS or the indicated ligand. The precipitates were analyzed by SDS-PAGE.

proteins of HIV-1 isolates with distinct tropism have been reported (3a, 18, 52, 59).

Representation of conformational states in populations of unliganded HIV-1 envelope glycoproteins. To investigate further the relationships among the different conformational states sampled by the HIV-1 envelope glycoproteins, untreated or cross-linked HIV-1 gp120 and sgp140(-/GCN4) envelope glycoproteins were repeatedly precipitated by individual ligands. As shown in Fig. 5, four successive immunoprecipitations with each of the ligands were sufficient to remove all of the envelope glycoprotein precipitable by the particular ligand. The supernatants containing the remaining envelope glycoproteins were divided and used for precipitation by the other ligands. All of the untreated sgp140(-/GCN4) glycoproteins could be precipitated by the ligands tested (Fig. 5B, left side). By contrast, a fraction of the untreated gp120 glycoprotein could only be precipitated by PS or the 39F anti-V3 antibody (Fig. 5A, left side). Upon fixation by cross-linking, relationships among the conformational states recognized by the gp120 ligands became apparent (Fig. 5, right half of each panel). For example, the b12 and 39F antibodies recognized all of the sgp140(-/GCN4) glycoprotein that could be precipitated by the F105 and 15e CD4BS antibodies but the F105 and 15e antibodies recognized only a fraction of the sgp140(-/GCN4) conformations precipitated by the b12 and 39F antibodies. The deduced relationships among the different conformations in the gp120 or sgp140(-/GCN4) glycoprotein are summarized in the diagrams in Fig. 6. These results indicate that monomeric gp120 and sgp140(-/GCN4) sample multiple conformations and that the conformations assumed by these unliganded glycoproteins are similar, although not necessarily identical.

Kinetics of ligand binding to soluble and cell surface HIV-1 envelope glycoproteins. The above analysis indicates that po-

tent neutralizing antibodies such as b12 and 39F recognize a large percentage of the conformations spontaneously sampled by the monomeric and trimeric HIV-1 envelope glycoproteins. This ability might give these antibodies a kinetic advantage, in that time delays associated with movement of the envelope glycoproteins into conformations able to be recognized by these ligands are eliminated, compared with those associated with ligands that recognize fewer possible envelope glycoprotein conformations. We first tested this hypothesis by using experimental conditions in which the HIV-1 envelope glycoproteins were free in solution and able to sample various conformations; to this end, the gp120 and sgp140(-/GCN4) envelope glycoproteins were precipitated by incubation with several of the ligands for various time periods. As shown in Fig. 7A and B, both b12 and 39F precipitated the gp120 and sgp140(-/GCN4) glycoproteins more efficiently after incubations of 15 min compared with CD4-Ig and the less potent neutralizing antibodies F105 and 15e. Even though b12 did not recognize the HIV-1 strain YU2 gp120 monomer as efficiently as some of the other antibodies after a 16-h incubation, after a short (15-min) incubation, b12 had already achieved a saturating level of binding whereas F105, 15e, and CD4-Ig required much longer time periods. These results indicate that b12 and 39F have kinetic advantages over the less potent neutralizing antibodies in recognizing soluble HIV-1 envelope glycoproteins.

We also examined the effect of incubation time on the binding of CD4-Ig and antibodies to the mature HIV-1 envelope glycoproteins on the surface of chronically infected cells, in the absence of cross-linking. As the wash and antibody detection steps are identical for all ligands in this experimental design, differences in the slope of the bound ligand versus time curves reflect differences in the rates with which the ligands associate

FIG. 6. Relationships among the conformational states of the unliganded HIV-1 envelope glycoproteins. The data shown in Fig. 5 were quantified and used to construct maps depicting the relationships among the conformational states sampled by the unliganded sgp140(-/GCN4) and gp120 envelope glycoproteins of HIV-1. The conformational states within each circle are able to be recognized by the indicated ligand.

initially with the envelope glycoproteins. Figure 7C shows that, compared with the F105 and 15e CD4BS antibodies, the more potently neutralizing b12 CD4BS antibody bound the cell surface HIV-1 envelope glycoproteins faster. Likewise, the potently neutralizing 2G12 antibody rapidly bound the mature HIV-1 envelope glycoproteins on the cell surface. The anti-V3 antibody and the 17b CD4i antibody, which recognize different elements of the chemokine receptor-binding moiety on gp120, exhibited slow kinetics of binding to the cell surface HIV-1 envelope glycoproteins. Of interest, CD4-Ig exhibited fast binding to the HIV-1 envelope glycoproteins expressed on the cell surface. Thus, in an experimental setting in which the initial rate of association makes a large contribution to the measured binding, CD4-Ig and the potent neutralizing antibodies b12 and 2G12 exhibited greater efficiency in binding the mature HIV-1 envelope glycoprotein trimers than the other gp120 ligands tested.

DISCUSSION

Thermodynamic studies suggest that the free, unliganded HIV-1 gp120 glycoprotein has a high entropy, which undergoes large decreases upon binding of ligands such as soluble CD4 or certain antibodies directed against the receptor-binding sites (30, 41). The high entropy of gp120 has been interpreted to reflect conformational flexibility in the free envelope glycoprotein (30, 41). Here we used the efficient cross-linker GA to investigate the conformational states of the free HIV-1 gp120 glycoprotein. As GA targets amines on the protein component of the glycoprotein (12, 34, 60), any observed decreases in antibody recognition of cross-linked envelope glycoproteins result from either fixation of the protein or modification of individual lysine residues important to the epitope. We observed that GA cross-linking affects the maximal amount of the HIV-1 envelope glycoproteins able to be recognized by antibodies, with minimal effects on the affinity of the antibody for the fraction of envelope glycoproteins still recognized. The fraction of the HIV-1 gp120 glycoprotein population able to be bound after cross-linking was a property of each antibody. These observations contrast with those expected for epitope

disruption, where decreases in affinity should be overcome by increasing antibody concentrations. The similarity of the results obtained in several instances for two different antibodies recognizing related but distinct epitopes also minimizes the potential contribution of epitope disruption by the cross-linker. Finally, the close relationship between relative ligand recognition of GA-cross-linked gp120 and the $-T\Delta S$ values for ligand-gp120 binding suggests that the impact of GA cross-linking on global conformational flexibility outweighs any direct detrimental effect of GA on epitope recognition for this set of antibodies. Thus, our data are not compatible with models in which a single unliganded gp120 conformation exists and the observed decreases in antibody recognition of cross-linked gp120 are due either to epitope disruption by GA or to a decreased ability of cross-linked gp120 to negotiate the conformational transitions required for antibody binding. Rather, our results support the existence of multiple conformations of the unliganded HIV-1 gp120 glycoprotein; the limitation of the conformational freedom of the protein component of gp120 largely accounts for the entropy changes observed upon binding of some ligands.

We compared the cross-linking results obtained for the gp120 monomer with those obtained for soluble, cleavage-defective envelope glycoprotein trimers and for cell surface cleaved envelope glycoprotein trimers. Our results provide an initial survey of the conformational states sampled by the free monomeric and trimeric HIV-1 envelope glycoproteins. Because cross-linking may not absolutely fix the conformation of all of the gp120 molecules in the population and because some ligands may recognize more than one conformation, our approach may overestimate the overlap that exists between two conformational states. Nonetheless, cross-linking analysis provides a conservative estimate of the number of HIV-1 envelope glycoprotein conformations. Notably, the CD4-bound state is occupied infrequently on the free HIV-1 envelope glycoproteins, consistent with the large amount of ordering and the associated unfavorable decrease in entropy associated with the transition to this state from the unliganded state (30, 41). This is also consistent with the poor abilities of HIV-1 gp120 to bind

FIG. 7. Kinetics of interactions of soluble and cell surface HIV-1 envelope glycoproteins with ligands. (A, B) Mixed radiolabeled supernatants containing gp120 and sgp140(-/GCN4) envelope glycoproteins were incubated at 4°C with the ligand-coupled protein A beads shown for the indicated time periods. The precipitates were analyzed by SDS-PAGE (A). The recognition of the sgp140(-/GCN4) and gp120 glycoproteins was quantified and is shown in panel B as a percentage of the respective glycoprotein precipitated by PS. (C) HIV-1 NL4-3(YU2)-infected PM1 cells were incubated at room temperature with the ligands shown for the indicated time periods. The cells were washed twice and incubated with the FITC-conjugated secondary antibody against human IgG before being subjected to FACS analysis. The geometric mean fluorescence intensity (MFI) is shown for each incubation time. The results shown are typical of those obtained in independent experiments.

the chemokine receptors in the absence of CD4 and to support CD4-independent infection (54, 61). The CD4i (17b, E51) antibodies recognize a gp120 conformation close to that of the CD4-bound state (30, 41) and also recognize only a small

fraction of the fixed envelope glycoproteins. The CD4BS antibodies F105 and 15e recognize conformations that are occupied by 20 to 35% of the unliganded soluble envelope glycoprotein molecules and perhaps an even lower percentage of

the mature cell surface envelope glycoproteins. Previous studies suggested that these CD4BS antibodies recognize HIV-1 gp120 conformations distinct from that of the CD4-bound state (63, 67). For the reasons discussed above, the overlap between these conformational states implied by the cross-linking analysis may simply reflect the resolution limits of this approach. The unique CD4BS antibody b12 and the 39F and 2G12 antibodies can recognize a majority of the conformations available to the free envelope glycoproteins. This characteristic correlates with the small entropic change that accompanies the binding of gp120 by these antibodies (30); these antibodies must bind in a way that does not limit the ability of gp120 to sample multiple conformational states. All three of these antibodies are thought to bind different elements of the gp120 outer domain, which presumably can move with respect to the other envelope glycoprotein domains but exhibits only minimal intradomain flexibility.

The low entropic change upon binding gp120, the recognition of a large fraction of the envelope glycoprotein conformational states, and potency of neutralization are all correlated properties of the b12, 39F, and 2G12 antibodies. Although envelope glycoprotein-directed neutralizing antibodies must interact with the functional trimeric viral spike, correlations between neutralizing potency and antibody binding to cell surface or viral envelope glycoproteins are not always apparent (20, 21, 41a, 41b, 43, 48, 51, 69). Our results demonstrate that, at equilibrium, only small differences exist between the binding of potent and weak CD4BS antibodies to the trimeric HIV-1 envelope glycoproteins; presumably, given sufficient time, the flexible envelope glycoproteins can conform to the shape recognized by the antibody, which in these experiments is present in excess. Under these conditions, any associated unfavorable entropic changes are offset by the enthalpy changes resulting from bond formation within gp120 and between the antibody and gp120 (30). Recognition of the fixed trimers by the antibodies was a much better indicator of neutralizing potency than equilibrium binding of the antibodies to the free trimers. This implies that neutralization of viruses, even in *in vitro* assays where virus and antibody are premixed, does not occur under conditions of equilibrium between virus and antibody. That the kinetics of antibody-virus interaction determine neutralizing efficacy is supported by the observation that the ability of an antibody to neutralize HIV-1 was predicted better by its on-rate constant than its equilibrium dissociation constant (48). If only a limited temporal opportunity for neutralization exists, an antibody that recognizes all of the conformations of the envelope glycoprotein has a distinct advantage over an antibody that can only recognize a fraction of the viral envelope glycoproteins at any given instant. To bind the viral envelope glycoprotein trimers to achieve neutralization, the latter antibodies must await conformational transitions that, although thermodynamically feasible, may occur with slow kinetics. High activation energy barriers between the conformational states of the functional trimer would slow the rate at which the envelope glycoproteins conform to the preferred shape for antibody binding. The delay in achieving a high-affinity interaction with antibody creates opportunities for competing interactions that lead to infection, such as CD4 binding, to occur. The ability to recognize the multiple conformations of the HIV-1 envelope glycoproteins may also be critical for successful drugs targeting gp120.

CD4 causes a large entropy decrease upon binding HIV-1 gp120 (41) and hypothetically encounters some of the impediments to binding facing weakly neutralizing antibodies. However, unlike antibodies, cell surface CD4 can bind multivalently to the HIV-1 envelope glycoprotein spike (41). The resulting increase in avidity may compensate for any decrease in affinity resulting from entropic barriers. Moreover, in this study, we observed that CD4-Ig exhibits faster binding to cell surface HIV-1 envelope glycoproteins than most antibodies that bind gp120 with comparably large entropic changes. CD4 may be able to initiate binding to gp120 molecules in many conformations, whereas antibodies that induce large conformational changes in gp120 may need to wait for the envelope glycoproteins to achieve the relevant conformation spontaneously. Thus, CD4 may have kinetic advantages over all but the most potent neutralizing antibodies that can rapidly bind many gp120 conformations.

Our results argue against a simple two-state model for the HIV-1 envelope glycoproteins (9), where CD4 binding drives the transition from a single unliganded conformation to the CD4-bound state. Further work is required to determine whether the structure of the unliganded SIV gp120 core (9) represents one of the free states of the functional HIV-1 envelope glycoproteins.

We observed that a substantial fraction of the soluble gp120 glycoprotein produced transiently after 293T cell transfection was not recognized efficiently by any conformation-dependent monoclonal antibody but was precipitated by the 39F anti-V3 loop antibody and by the PS from HIV-1-infected individuals. This serum pool can bind denatured gp120, so this fraction of gp120 may be in a nonnative conformation. As these unknown states of gp120 can be observed in the absence of cross-linker, this subset of gp120 molecules appears to be naturally trapped, unable to make the transition to a form precipitable by known conformation-dependent monoclonal antibodies. In the absence of cross-linker, these gp120 variants migrate faster than the unselected gp120 population, so they may represent underglycosylated molecules that assume nonnative folds during the process of overexpression in the 293T cells. Such forms were not apparent for the soluble trimeric HIV-1 envelope glycoproteins.

After cross-linking, certain epitopes on soluble or cell surface envelope glycoprotein trimers were recognized less efficiently than the same epitopes on cross-linked monomeric gp120. The relative recognition of the fixed versus untreated trimers by the 2/11c and C11 antibodies was less than that observed for the gp120 monomer. This is consistent with the expectation that these antibodies recognize gp120 regions that are involved in the noncovalent interaction with the gp41 ectodomain (40, 62). Binding of the 39F antibody to the V3 loop of cross-linked cell surface envelope glycoproteins was very low, even though the 39F antibody efficiently recognized the soluble gp120 or sgp140(–/GCN4) glycoproteins after GA treatment. The differences between soluble trimers and cell surface trimers may be due to differences in proteolytic processing, membrane anchorage, and/or subunit interactions.

The cross-linked sgp140(–/GCN4) trimers appeared to mimic the functional HIV-1 envelope glycoproteins with re-

spect to the expected pattern of epitope exposure. Potent and weak neutralizing antibodies, as well as nonneutralizing antibodies, could readily be distinguished on the basis of recognition of the cross-linked envelope glycoprotein trimers. This characteristic of the cross-linked sgp140(-/GCN4) trimers may make them useful for screening for potent HIV-1-neutralizing monoclonal antibodies, for understanding differences among HIV-1 strains in neutralization sensitivity, and for designing immunogens.

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