2007

Characterization of Human Immunodeficiency Virus Type 1 Monomeric and Trimeric gp120 Glycoproteins Stabilized in the CD4-Bound State: Antigenicity, Biophysics, and Immunogenicity

Barna Dey
Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland

Marie Pancera
Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland

Krisha Svehla
Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland

Yuuei Shu
Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland

Shi-Hua Xiang
University of Nebraska-Lincoln, sxiang2@unl.edu

See next page for additional authors

JOURNAL OF VIROLOGY, June 2007, p. 5579–5593 Vol. 81, No. 11
0022-538X/07/$08.00+0 doi:10.1128/JVI.02500-06

Characterization of Human Immunodeficiency Virus Type 1 Monomeric and Trimeric gp120 Glycoproteins Stabilized in the CD4-Bound State: Antigenicity, Biophysics, and Immunogenicity▽

Barna Dey,[1] Marie Pancera,[1] Krisha Svehla,[1] Yuuei Shu,[1] Shi-Hua Xiang,[2] Jeffrey Vainshtein,[2] Yuxing Li,[1] Joseph Sodroski,[2] Peter D. Kwong,[1] John R. Mascola,[1] and Richard Wyatt[1]*

Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892,[1] and Dana-Farber Cancer Institute, Boston, Massachusetts 02115[2]

Received 13 November 2006/Accepted 23 February 2007

The human immunodeficiency virus type 1 exterior gp120 envelope glycoprotein is highly flexible, and this flexibility may contribute to the inability of monomeric gp120 immunogens to elicit broadly neutralizing antibodies. We previously showed that an S375W modification of a critical interfacial cavity central to the primary receptor binding site, the Phe43 cavity, stabilizes gp120 into the CD4-bound state. However, the immunological effects of this cavity-altering replacement were never tested. Subsequently, we screened other mutations that, along with the S375W alteration, might further stabilize the CD4-bound state. Here, we define a selected second cavity-altering replacement, T257S, and analyze the double mutations in several gp120 envelope glycoprotein contexts. The gp120 glycoproteins with the T257S-plus-S375W double mutation (T257S+S375W) have a superior antigenic profile compared to the originally identified single S375W replacement in terms of enhanced recognition by the broadly neutralizing CD4 binding-site antibody b12. Isothermal titration calorimetry measuring the entropy of the gp120 interaction with CD4 indicated that the double mutant was also stabilized into the CD4-bound state, with increasing relative fixation between core, full-length monomeric, and full-length trimeric versions of gp120. A significant increase in gp120 affinity for CD4 was also observed for the cavity-filling mutants relative to wild-type gp120. The most conformationally constrained T257S+S375W trimeric gp120 proteins were selected for immunogenicity analysis in rabbits and displayed a trend of improvement relative to their wild-type counterparts in terms of eliciting neutralizing antibodies. Together, the results suggest that conformational stabilization may improve the ability of gp120 to elicit neutralizing antibodies.

The human immunodeficiency virus type 1 (HIV-1) envelope glycoproteins gp120 and gp41 form a specialized type I viral membrane fusion complex that mediates viral entry (9). The gp120 glycoprotein is composed of two major structural domains which contribute to the receptor-binding elements that interact with the viral receptors CD4 and CCR5/CXCR4 (1, 8, 11, 13, 14, 16). HIV-1 gp41 contains the trimerization domain and mediates viral-to-target-cell membrane fusion (17). Because the trimeric glycoprotein spike mediates receptor binding and is the only viral gene product on the surface of HIV, it is the sole target for virus-specific neutralizing antibodies. Attempts to elicit broadly neutralizing antibodies using monomeric gp120 (2, 3, 4, 10, 26, 44), peptide regions from gp41 (27, 31, 36), or trimeric soluble gp140 mimics of the envelope spike (15, 19, 24, 38, 48) were met with limited success. Therefore, we sought novel and alternative means to achieve this goal.

We suggested previously that the extreme flexibility of gp120 may present many conformations to the humoral immune system not found on the functional spike and may contribute to its tendency to elicit antibodies that bind to monomeric gp120 but rarely broadly neutralizing antibodies (30, 47). Broadly neutralizing antibodies presumably bind relatively efficiently to conserved regions of the functional spike that are shrouded by glycan and immunodominant variable elements, hence rendering them poorly immunogenic. Binding antibodies, specifically those that can bind to conserved gp120 elements but not the functional spike, recognize "open" conformations of gp120 that are not accommodated on the spike due to conformational masking (21) or nonneutralizing determinants involved in trimer contacts. Hence, neither of these types of epitopes is exposed on the assembled, constrained quaternary structure of the functional spike. Therefore, as an avenue of immunogen development, we sought means to lock gp120 into a constrained conformation that is known to exist on the functional spike. One such conformation, highly relevant to the entry process, which needs to be sampled by the functional spike for entry and fusion, is the CD4-bound conformation of gp120. Normally, CD4 is required to induce this conformation (22, 30). Here, we have extended our attempts to generate the CD4 state of gp120 by a mutagenic approach as described previously (47). Guided by the X-ray crystal structure of the ternary complex of gp120, CD4, and the chemokine receptor mimetic antibody 17b, we demonstrated that a single serine (S)-to-tryptophan (W) replacement of amino acid 375 in a region described as the "Phe43 cavity" significantly stabilized gp120 into the CD4-bound conformation (22, 47). The Phe43 cavity lies at the nexus of the gp120 inner domain, outer domain, and bridging sheet and is proximal to critical contacts with the primary receptor, CD4. Besides restricting the conformational flexibility of gp120, the cavity-filling mutation increases CD4

* Corresponding author. Mailing address: Vaccine Research Center, National Institutes of Health, 40 Convent Drive, Rm. 4512, Bethesda, MD 20892. Phone: (301) 594-8690. Fax: (301) 480-0274. E-mail: richardwyatt@nih.gov.

▽ Published ahead of print on 14 March 2007.

and 17b recognition slightly while eliminating recognition by several nonneutralizing CD4 binding-site-directed (CD4BS) antibodies. Conversely, other mutations in the bridging sheet could eliminate recognition by CD4 while retaining recognition by the CD4BS antibodies. These data suggested a model by which the cavity mutation locked gp120 in a conformation favorable for CD4 and 17b recognition (and hence CCR5 interaction and entry) but not for recognition by the nonneutralizing CD4BS antibodies (47). Hence, we termed the cavity-filled gp120 proteins to be in the "CD4 state" even though entropy analysis indicated that the conformational alteration, although substantial, was not absolute. To further lock gp120 into the CD4-17b-bound conformation, or CD4 state, we analyzed a set of second-site mutations based upon the following criteria. The first subset was designed to relieve a potential clash of the bulky 375W residue with residue T257 in the Phe43 cavity. The second subset of noncavity mutations were introduced since they, by themselves, cause decreases in recognition by the CD4BS antibodies without affecting CD4 recognition greatly as previously reported (40, 41, 42). Since 17b and CD4 recognize the same conformation of gp120 and since CD4 induces high-affinity binding of gp120 to CCR5, we ranked the double mutants based upon 17b and CCR5 recognition in the absence of CD4. From these data and technical issues of protein solubility following deglycosylation, we selected a second cavity-altering mutation, T257S, in combination with the S375W change (T257S+S375W), for further antigenic and biophysical analyses. Several gp120 contexts were tested to determine the best candidate to analyze the impact of the cavity-filling, CD4-state mutations on the elicitation of neutralizing antibodies.

As previously reported, although the S375W mutation eliminated the recognition of gp120 by several nonneutralizing CD4BS antibodies, it also adversely affected recognition by the broadly neutralizing CD4BS antibody b12 (47). Due to the neutralization breadth and potency of the b12 antibody, we reasoned that it is likely desirable to retain the b12 epitope in a gp120-based immunogen design (6). Somewhat fortuitously, when the T257S replacement was introduced along with the S375W CD4-state mutation, b12 recognition was increased, while protein stabilization was maintained.

Given this improved protein design, we sought to test the hypothesis that the stabilization of gp120 in the CD4-bound conformation would provide an immunological benefit in eliciting broadly cross-reactive neutralizing antibodies. In this study, we report the antigenic, biochemical, and biophysical characterization of cavity-altered double mutant proteins in monomeric contexts (core and full-length gp120) and in the recently characterized trimeric gp120-GCN4 context (32). Because the cavity alterations had the greatest degree of stabilization in the trimeric gp120 context, we assessed the ability of the stabilized soluble gp120 spike mimetics to elicit neutralizing antibodies relative to wild-type (WT) monomeric and trimeric gp120 glycoproteins.

MATERIALS AND METHODS

Cells and proteins. HEK293 cells were obtained from the American Type Culture Collection. Soluble human D1D2 CD4 was obtained through the NIH AIDS Reference and Reagent Program, Division of AIDS, NIAID, NIH (18). The monoclonal antibodies b12 and b6 used in this study were engineered as immunoglobulin G1 constructs as previously described (6) and were generous gifts from Dennis Burton, and F105 (34, 35) was kindly provided by Marshal Posner. 2G12 (43) and 2F5 (29) were kind gifts from Herman Katinger. The 17b antibody (41) and the biotinylated guinea pig IgG anti-CD4 were kindly provided by James Robinson.

Construction of Env expression plasmids. The unmodified YU2 core expression plasmid pBD103 was derived from a previously described YU2 gp120ΔV1V2V3-GCN4 construct (32) by the deletion of the N terminus (amino acids 1 to 81) and the C terminus (amino acid 497 onward) and by the introduction of a stop codon upstream of the GCN4 trimerization sequence. The mutant core protein with Phe43 cavity-filling mutations was expressed from plasmid pBD105 by altering two amino acid residues at positions 375 (from serine to tryptophan) and 257 (from threonine to serine) in pBD103. As previously described, monomeric and trimeric WT gp120 proteins were expressed from plasmids encoding WT YU2 gp120 monomers and YU2 gp120-GCN4 trimers, respectively (32). Codon changes to introduce S375W and T257S mutations into the YU2 gp120 monomeric WT and YU2 gp120-GCN4 plasmids for expressing monomeric and trimeric mutant gp120 proteins then generated plasmids pBD202M and pBD202T, respectively. Generation of the cysteine-constrained HXBc2 core Ds12F123 construct was described elsewhere previously (49). All the above-mentioned modifications were made by QuikChange mutagenesis (Stratagene) in pcDNA3.1-based constructs and confirmed by sequencing. Site-directed mutagenesis was also used to introduce second-site replacements into the previously described YU2 375S/W protein containing deletions of N-terminal gp120 residues 31 to 81 and V1/V2 residues 128 to 194 (47). For making WT pseudotyped YU2 virus, the open reading frame of the YU2 gp160 protein was inserted into KpnI and BamHI sites of vector pSVIIIenv to generate an Env expressor plasmid (39). Site-directed mutagenesis was performed to introduce an S375W and/or a T257S mutation to generate the mutant Env expressor plasmids. In all cases, the numbering of gp120 amino acid residues is based on the sequence of the prototypic HXBc2 strain (20).

Molecular modeling. Designing an S375W mutation in the context of a truncated YU2 gp120 protein was described previously (47). The modeling suggested a potential clash between the bulky tryptophan side chain and the C-gamma atom of threonine 257 that might have an impact on core gp120 protein stability. Therefore, a second mutation changing threonine to serine at position 257 was designed to alleviate the potential clash. The mutation was introduced into core gp120, full-length monomeric gp120, and full-length trimeric gp120 molecules. The rationale for modeling other second-site replacements to enhance the CD4-bound conformation is found in Table 1.

Expression and purification of monomeric and trimeric envelope glycoproteins. All proteins were expressed in serum-free medium by transient transfection of HEK293T cells. Briefly, HEK293T cells growing in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum, 2 mM glutamine, and 1× penicillin-streptomycin (50 units/ml penicillin, 50 μg/ml streptomycin) were seeded at a density of 1.2×10^7 cells per 150-cm^2 tissue culture dish. After overnight incubation, cells were transfected with a mixture of expressor plasmid and the transfection reagent Fugene (Roche) according to the manufacturer's instructions. The following day, transfection medium was replaced with 293 SFMII (serum-free medium) supplemented with 4 mM glutamine. Four days later, culture supernatants were collected, centrifuged at $3{,}500 \times g$ to remove cell debris, filtered through sterile 0.2-μm filters, and stored at 4°C (for a maximum of up to 2 weeks) in the presence of protease inhibitors prior to purification. All proteins were purified over affinity columns, and in selected cases, the trimeric fractions were isolated by size exclusion chromatography. The F105 (34, 35) affinity column was used for the purification of unmodified core and WT gp120 trimer proteins. Either an F105 or a b12 (6) affinity column was used for WT gp120 monomer protein purification, and a 17b (41) affinity column was used to purify all mutant proteins. For purification of monomeric proteins, the culture supernatant was applied to the affinity column overnight at room temperature (RT), and the column was washed with 10 volumes of phosphate-buffered saline (PBS) (pH 7.4) containing 0.5 M NaCl and washed with 5 volumes of PBS containing 0.15 M NaCl, eluted with 100 mM glycine (pH 2.8), and the eluate was immediately neutralized to pH 7.0 with 1 M Tris base. Trimeric proteins were eluted with 3 M $MgCl_2$ prepared in 20 mM Tris-HCl (pH 7.4). For all proteins, eluted fractions containing proteins, as determined by the optical density (OD) at 280 nm, were pooled, concentrated with Amicon Ultra centrifugal filter devices (Millipore, Bedford, MA), and dialyzed extensively against PBS (pH 7.4). Protein aliquots were flash-frozen in liquid nitrogen and stored at −80°C until further use.

Affinity-purified trimeric proteins were further subjected to size exclusion chromatography using a Superdex 200 16/26 column (Amersham Pharmacia) in PBS containing 0.35 M NaCl. The flow rate was set to 1 ml/min for the first 100 min and reduced to 0.5 ml/min until the end of the run, which allowed the

TABLE 1. Second-site mutants in combination with the 375S/W CD4-state mutation[a]

gp120 position[b]	Introduced change	Description	Relative binding[c]	
			17b	CCR5
None			1.0	1.0
257	T/A	Removes potential steric clash with Trp375	0.8	0.5
	T/M	Should exacerbate steric clash with Trp375	0.3	0.3
	T/S	Removes steric clash while maintaining important side chain hydroxyl H bond at position 257	1.1	1.1
266	A/E	Reduces binding for CD4BS antibodies 15e, 21h, and 1125h	1.15	0.7
386	N/Q	Decreased recognition of CD4BS antibodies 15e, 21h, and 1125h	0.85	0.9
429	E/F	Enhances overall hydrophobic stability of the bridging sheet	0.85	0.5
435	Y/W	Enhances hydrophobic interactions between the bridging sheet and Phe382 of the outer domain	0.55	0.00
470	P/G	Decreased CD4BS antibody recognition while maintaining CD4 binding	0.4	0.4

[a] The rationale was to lock gp120 into the conformation recognized by CD4 and 17b, a 375S/W replacement in the gp120 Phe43 cavity that increased the binding affinity of gp120 to CD4 and reduced binding to several CD4BS antibodies (47). The mutation also increased binding to CD4i antibody 17b and to the chemokine receptor CCR5, which was used here to select the T257S mutant for further analysis.

[b] Second-site mutations were incorporated into the previously described 375S/W gp120 protein (47).

[c] Radiolabeled soluble envelope glycoproteins were produced by transient transfection of 293T cells and analyzed for recognition by multiple ligands in immunoprecipitation assays as described previously (47). The amount of precipitated mutant protein relative to that of the WT protein was determined by PhosphorImager analysis (Molecular Dynamics) and converted to a relative scale where YU2 375S/W recognition by 17b and CCR5 in the absence of CD4 was equal to 1.00.

separation of the oligomeric species. Relevant fractions were pooled, concentrated, dialyzed against PBS (pH 7.4), flash-frozen, and stored at −80°C.

Native gel analysis. Before and after size exclusion chromatography, monomeric and trimeric gp120 proteins were run on blue native gels to analyze the purity of the oligomeric states. The running buffer for the outer chamber contained 50 mM Tris-HCl plus 50 mM MOPS (morpholinepropanesulfonic acid) (pH 7.7). The inner chamber buffer also contained 0.001% of SERVA Blue G (catalog no. 35050). Protein samples were diluted 1:1 in 2× sample buffer (100 mM Tris-HCl, 100 mM MOPS, 40% glycerol, 0.1% Serva-Blue G [pH 7.7]) and run for 4 h at 4°C at 100 mV. Standard molecular weight markers were included in the analysis. Following electrophoresis, gels were stained with Coomassie blue and then destained according to standard procedures used for sodium dodecyl sulfate (SDS)-containing gels.

Immunoprecipitation. CD4 induction of gp120 proteins for 17b binding was measured by immunoprecipitation assays as follows. In separate microcentrifuge tubes, 1 ml of culture supernatant was incubated without or with 10 μg of soluble CD4 (sCD4) (human D1D2) for 30 min at RT. Ten micrograms of 17b and 25 μl of a 50% suspension of Sepharose-protein A beads were then added to each reaction mixture and incubated for 1 h at RT. The beads were then pelleted by centrifugation at 5,000 rpm for 2 min and washed twice with PBS (pH 7.4) containing 0.5 M NaCl and twice with PBS (pH 7.4). Each pellet was then resuspended in 30 μl of reducing SDS sample buffer and heated to 100°C for 5 min. The contents were mixed by vortexing and centrifuged at 10,000 rpm for 2 min to pellet the beads. The supernatant fractions were then carefully subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and Coomassie staining using standard procedures.

ELISA. The antigenicities of WT and mutant envelope proteins were determined by standard a enzyme-linked immunosorbent assay (ELISA) as follows. Corning high-protein-binding ELISA plates were coated with 400 ng per well of *Galanthus nivalis* lectin (catalog no. L8275-5MG; Sigma) in 100 μl of PBS (pH 7.4) at 4°C overnight. The lectin was then removed, and the wells were blocked for 3 h at RT with PBS–2% fat-free milk and 4% fetal calf serum, followed by five washes with wash buffer (PBS–0.2% Tween 20). The wells were then incubated with 200 ng of protein in 100 μl of PBS for 2 h at RT, followed by five washes and incubation with 100 μl of different anti-gp120 monoclonal antibody solutions that were fivefold serially diluted starting with 20 μg/ml of the initial concentration in dilution buffer (1:10-diluted blocking buffer). Following 1 h of incubation at RT, the wells were washed and incubated for 1 h at RT with 100 μl of a horseradish peroxidase (HRP)-conjugated anti-human IgG (catalog no. 109-036-097; Jackson ImmunoResearch Laboratories, Inc.) solution at a 1:10,000 dilution in antibody dilution buffer. After five washes, 100 μl of the colorimetric peroxide enzyme immunoassay substrate (3,3′,5,5′-tetramethylbenzidine; Bio-Rad) was added to each well, and the reaction was stopped by adding 100 μl of 1 M sulfuric acid to the mixture. The OD of the wells was read at 450 nm using an ELISA plate reader. All samples were run in duplicates. The average OD of negative control wells containing bovine serum albumin (BSA) was subtracted from the average OD of experimental wells to obtain the final OD values. For the ELISA of unmodified and mutant YU2 core proteins shown in Fig. 1B (left panels) and for the analyses described in the legend of Fig. 8, 200 ng of proteins was directly coated onto each well of the ELISA plates.

To determine the anti-gp120 antibody titers in immunized sera (see Table 3), ELISA plates were coated with 200 ng of WT YU2 gp120 monomeric protein in 100 μl of PBS per well. After blocking and washes, fivefold serial dilutions (starting at 1/200) of the sera from immunized rabbits were added in duplicate wells and incubated for 2 h at RT. Following washes, the wells were incubated with a 1:10,000 dilution of HRP-conjugated anti-rabbit IgG (catalog no. 111-035-046; Jackson ImmunoResearch Laboratories, Inc.) and developed with HRP substrate, and the ODs were read at 450 nm.

For characterizing the binding properties of antibodies elicited by the three different immunogens used in this study (as shown in Fig. 7), ELISA plates were coated with 100 μl of unmodified core protein (2 μg/ml) and incubated with fivefold serial dilutions (starting at 1/200) of the immune sera (following four inoculations at a 125-μg dosage) in 100 μl of 1:5-diluted blocking buffer. After 30 min at RT, 100 μl of D1D2 CD4 (0.8 μg/ml in 1:5-diluted blocking buffer) was added to each plate for 1 h at RT. The plates were then washed and incubated with a 1:2,500 dilution of biotinylated guinea pig IgG anti-CD4 for 1 h at RT. Following washes, the wells were incubated with 100 μl of a 1:200 dilution of streptavidin-peroxidase (S2438; Sigma) and developed with HRP substrate, and the ODs were read at 450 nm.

ITC. Isothermal titration calorimetry (ITC) was carried out using a VP-ITC titration calorimeter system from MicroCal, Inc. All proteins, including sCD4 (D1D2), were thoroughly dialyzed against PBS and degassed before use. The dialysis buffer was filtered through a 0.2-μm membrane and used to dilute the protein samples. The concentration of envelope protein in the sample cell was approximately 4 μM, and the concentration of sCD4 in the syringe was approximately 40 μM. The reference cell contained degassed Milli-Q water. Envelope glycoproteins in the sample cell were titrated to saturation by the stepwise addition of 10 μl of sCD4 from the syringe at 400-s intervals at 37°C. The heat evolved upon each injection of titrant (sCD4) was obtained from the integral of the calorimetric signal. The heat of dilution of sCD4 was subtracted from the heat of reaction with gp120 to obtain the heat associated with the binding reaction. The molar concentrations of the proteins were calculated using the following molar extinction coefficients: core, 1.22; mutant core, 1.38; WT gp120, 1.41; mutant gp120, 1.52; WT trimer, 1.4; mutant trimer, 1.38. The specific activity of sCD4 was determined to be approximately 80% as described previously (32). The values for enthalpy (ΔH), entropy (ΔS), and the association constant (K_a) were obtained by fitting the data to a nonlinear least-squares analysis with Origin software.

Immunization protocol. New Zealand White rabbits (females, approximately 12 weeks of age) were inoculated by the intradermal route with 125 μg of proteins emulsified in a 1:1 dilution of Ribi adjuvant (Corixa, Hamilton, MT) in a total volume of 1 ml. One inoculation of 500 μl each was administered in each hind leg. The protein-adjuvant emulsions were prepared within 2 h of inoculation into animals. Boosting inoculations were administered at 4-week intervals fol-

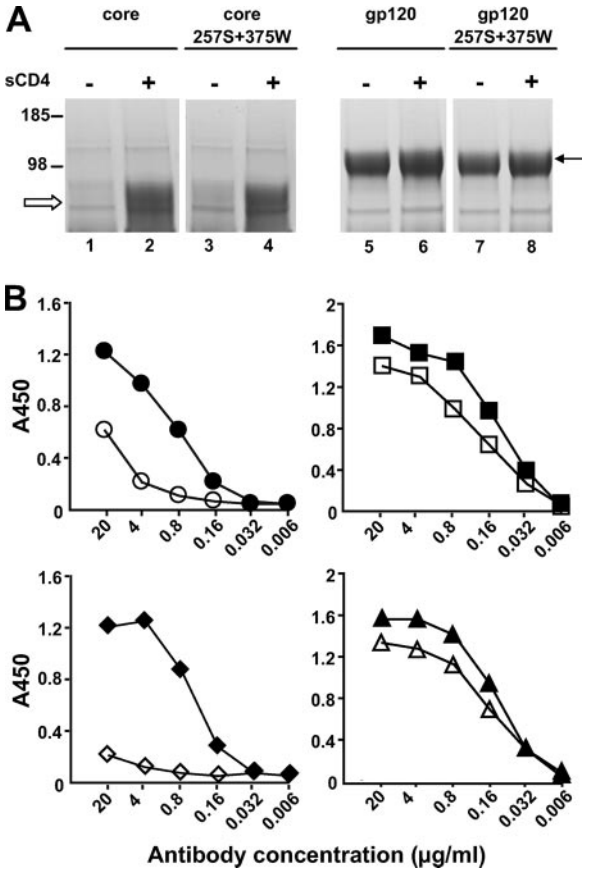


FIG. 1. Immunoprecipitation and ELISA analysis of WT and mutant (T257S+S375W) variants of core and gp120 proteins to characterize 17b binding in the presence or absence of sCD4. (A) Immunoprecipitation and gel analysis. Supernatants from transfection cultures of envelope glycoproteins were incubated with 17b monoclonal antibody and protein A-agarose beads with or without prior incubation with sCD4. The immunoprecipitated proteins were analyzed by SDS-PAGE and visualized with Coomassie blue staining. Lane 1, unmodified core without sCD4; lane 2, unmodified core with sCD4; lane 3, mutant core without sCD4; lane 4, mutant core with sCD4; lane 5, WT gp120 without sCD4; lane 6, WT gp120 with sCD4; lane 7, mutant gp120 without sCD4; lane 8, mutant gp120 with sCD4. Migration of core proteins (open arrow) and gp120 monomers (solid arrow) is indicated. (B) ELISA. Equal amounts of affinity-purified unmodified and mutant proteins were coated onto ELISA plates in duplicates and reacted with fivefold serial dilutions of 17b antibody in the presence (solid symbols) or absence (open symbols) of 20 μg/ml of sCD4. 17b binding to various Envs was detected by reacting the wells with anti-human IgG conjugated to peroxidase. Upper left (circles), WT core; lower left (diamonds), mutant core; upper right (squares), WT gp120; lower right (triangles), mutant gp120. Margins of error from duplicate reactions were negligible in all cases.

lowing the initial inoculation. Test bleeds were collected 7 to 10 days after each booster inoculation. For serum preparation, blood was incubated at RT for 2 h to allow clotting and centrifuged for 10 min at 2,000 × *g* to separate the liquid phase from the clotted components. The serum was heat inactivated at 56°C for 1 h and stored at −20°C for subsequent analysis.

Neutralization assays. HIV-1 was pseudotyped with selected envelope glycoproteins by the cotransfection of an *env* expressor and viral genomic DNA with a deletion of Env into 293T cells. Following the production of pseudotyped virus, a luciferase-based neutralization assay was performed as previously described (24). Briefly, TZM-bl cells expressing CD4, CXCR4, and CCR5 were used for HIV-1 infection. These target cells contain Tat-responsive reporter genes for firefly luciferase and the *Escherichia coli* β-galactosidase gene under the regulatory control of the HIV-1 long terminal repeat. The level of HIV-1 infection was quantified by measuring relative light units (RLU) of luminescence, which is directly proportional to the amount of virus input. The assays were performed using a 96-well microtiter plate format with 10,000 TZM-bl cells per well. The construction and preparation of pseudotyped viruses were described elsewhere previously (23). For neutralization assays, each pseudotyped virus stock was diluted to a level that produced approximately 100,000 to 500,000 RLU. The percentage of virus neutralization by each immune serum sample was derived by calculating the reduction in RLUs in the test wells compared to the RLUs in the wells containing preimmune serum from the corresponding animal. To control for nonspecific neutralization in protein-immunized rabbits, sera from two animals immunized with BSA were analyzed. All serum samples were also assayed for neutralizing activity against a pseudovirus expressing the amphotropic murine leukemia virus envelope to test for non-HIV-1-specific plasma effects (25).

Neutralization of HIV-2 strain 7312A/V434M was performed as previously described (12). Briefly, pseudovirus stock was treated with mock media or with 0.5 μg/ml of sCD4 (50% inhibitory concentration [IC_{50}] for entry of this virus) for 1 h before adding sera. The remainder of the assay was done as described above. To calculate the percent neutralization with sCD4 present in the assay, the baseline RLU was the value measured with virus plus sCD4 and no serum.

To obtain IC_{50} data, fivefold serial dilutions of immune sera were incubated with viruses before infection of target cells. Antiserum dose-response curves were fit with a nonlinear function, and the IC_{50} for the corresponding virus was calculated by a least-squares regression analysis. Statistical analysis of the IC_{50} titers was performed with the unpaired *t* test (GraphPad Prism software package 3.0; GraphPad Software Inc., San Diego, CA).

Virus entry assay. WT and mutant pseudotyped YU2 viruses were produced by cotransfection of envelope glycoprotein expressor plasmids and viral genomic DNA with a deletion of the *env* gene into 293T cells. Pseudovirus titers were adjusted by p24 ELISA (Beckman Coulter) according to the manufacturer's protocol. Equivalent doses of virus suspended in a 40-μl volume were then mixed with 20 μl of TZM-bl cells (10,000 cells) and 10 μl of medium on 96-well plates and incubated overnight at 37°C. The following day, 130 μl of cell culture medium was added to each well and incubated for an additional 24 h. Cell culture medium then was removed from all wells, and 50 μl of cell lysis buffer (Promega, Madison, WI) was added. Thirty microliters of cell lysis supernatant was transferred onto a new plate containing substrate for the measurement of luminescence using a luminometer. The RLU produced by the wells were measured and used to calculate viral entry. To determine antibody-mediated neutralization of HIV-1 entry, each viral inoculum was preincubated with fourfold serial dilutions of antibody in 50 μl of medium for 1 h at 37°C. After virus-antibody incubation, the TZM-bl target cells were added to all wells. The initial concentrations of the antibodies tested were as follows: b12, 25 μg/ml (6); 2F5, 50 μg/ml (29); and HIV IgG (IgG pool from HIV-infected patient sera), 5 mg/ml.

IgG adsorption and elution assay. Equal volumes (150 μl) of rabbit sera were mixed separately with recombinant protein A-conjugated Sepharose Fast Flow beads (catalog no. 17-1279-02; Amersham) and unconjugated Sepharose 4B beads (catalog no. 4B200; Sigma) at a 1:1 ratio of serum to beads in microcentrifuge tubes and incubated for 1 h at RT with continuous mixing in a nutating mixer. The adsorbed sera were then separated by pelleting the beads for 3 min at 5,000 × *g*. The beads were washed twice with 1.2 ml PBS containing 0.5 M NaCl and once with PBS (0.15 M NaCl), and all liquid was removed from the top of the pellets. In order to elute captured IgG from the beads, each pellet was resuspended in 135 μl of 0.1 M glycine (pH 2.0) for 1 min at RT and then centrifuged at 500 × *g* for 3 min. The supernatant containing IgG was then separated, and the pH was neutralized by adding 15 μl of 1 M Tris (pH 8.5). The percent recovery of IgG was determined by ELISA, where fivefold serial dilutions of untreated sera as well as different fractions of treated sera were directly coated onto ELISA plates overnight at 4°C, which was followed by detection with HRP-conjugated goat anti-rabbit IgG and reading the absorbance at 450 nm.

RESULTS

Expression and immunoprecipitation analysis of gp120 variant proteins. We have shown previously that although the YU2 375S/W protein was poorly recognized by several non-neutralizing CD4BS antibodies, recognition by the potent and broadly neutralizing CD4BS antibody b12 was also compromised (47). Additionally, attempts to deglycosylate the HX core S375W protein for structural analysis decreased solubility to such an extent that crystallographic screening was not pos-

sible (T. Zhou et al., personal communication). The decreased solubility was attributed to a potential steric clash of the bulky tryptophan 375 side chain within the Phe43 cavity. Therefore, we screened a set of second-site mutations of the YU2 375S/W protein designed to alleviate this potential clash and to generate an antigenically and biochemically better-behaved molecule stabilized in the CD4 state. One subset of mutations was modeled to relieve a potential clash in the cavity or to enhance hydrophobic interactions of the bridging sheet subdomain with other elements of gp120 and were based upon the crystal structure. A second subset was selected from mutations studied previously that reduced CD4BS antibody recognition while maintaining CD4 binding or enhanced binding to 17b (or to CCR5) (40, 41, 42). Selected properties of the double mutations are listed in Table 1. Analysis of the panel of eight double mutations suggested that the combination of T257S and S375W had the best profile based upon CD4-independent recognition by 17b, binding to CCR5 in the absence of CD4, and relative CD4 recognition (Table 1 and data not shown). In addition, HX core proteins containing the cavity mutations at positions 257 and 375 expressed well and showed increased resistance to enzymatic deglycosylation as well as enhanced solubility following deglycosylation (Zhou et al., personal communication). To test the impact of the cavity-altering mutations eventually in immunogenicity, we introduced these mutations into plasmid DNA sequences encoding envelope glycoproteins derived from the neutralization-resistant primary isolate YU2. We focused on further analysis of the structurally defined YU2 core gp120 in presence of these mutations. For biological relevance and eventually immunogenicity, we included the analysis of the cavity-altering mutations in the context of full-length monomeric gp120 and in the context of the recently described soluble gp120 trimeric spike mimetics (32).

To confirm the structural integrity of the secreted monomeric and trimeric variants, we analyzed the recognition of proteins in tissue culture cell supernatants by immunoprecipitation with selected antibodies. Consistent with previous observations (41, 47), full-length gp120 and the full-length T257S-plus-S375W gp120 proteins were recognized well by the CD4-induced (CD4i) antibody 17b and were induced slightly in the presence of CD4 (Fig. 1, lanes 5 to 8). The same mutations generated in the trimeric gp120 context had an effect similar to that seen for gp120 (data not shown).

Unlike full-length gp120, core gp120 proteins bind poorly to 17b. The interaction of gp120 with CD4 induces conformational changes that allow more efficient 17b recognition of the core proteins. Therefore, 17b recognition of core proteins in the presence of CD4 is useful as an indicator of proper folding. Because 17b and CD4 are recognized by the same gp120 conformation as that determined in the ternary structure of the gp120-CD4-17b complex, we also assessed whether recognition of the mutant core proteins in the absence of CD4 might indicate the stabilization of gp120 in the CD4-bound conformation by the cavity-altering mutations. As shown in Fig. 1A, unmodified core protein was weakly recognized by 17b in the absence of CD4 (lane 1). As expected, 17b binding was greatly induced in the presence of CD4 (Fig. 1A, lane 2) and represents the maximal recognition of the core by 17b. A similar binding pattern was obtained with the cavity-altered core protein showing only a weak 17b interaction in the absence of CD4 (Fig. 1A, lane 3), but efficient induction of this interaction by CD4 achieved maximal 17b binding, which is equivalent to that of the unmodified protein (lane 4). These results indicated that although the mutant proteins were properly folded, the cavity mutations by themselves did not completely stabilize core protein in the CD4-bound conformation (Fig. 1A, lanes 3 and 4). Similar patterns of 17b binding in the presence and absence of CD4 were observed by ELISA (Fig. 1B) using affinity-purified proteins as described below.

Purification and binding analysis of the gp120 glycoproteins. The gp120 glycoproteins were purified over affinity columns and analyzed by gel electrophoresis. The migration of WT and mutant proteins under reducing condition is shown in Fig. 2A. Under these conditions, monomeric (Fig. 2A, lanes 3 and 4) and trimeric (lanes 5, 6, and 7) gp120 proteins exhibited similar mobilities, while core proteins were observed as a faster-migrating species. Differences in molecular weight between monomeric gp120 and the trimeric gp120-GCN4 variants were apparent by blue native gel analysis (Fig. 2B). Purification of proteins possessing the GCN4 trimerization motif by affinity chromatography revealed that the preparation contained some higher-molecular-weight species as shown in lane 4 of Fig. 2B. Homogeneous trimers were then separated from the higher-molecular-weight species by size exclusion chromatography (Fig. 2C). For the mutant trimers, gel filtration-purified trimer fractions were analyzed and purity was confirmed by blue native gels (Fig. 2B, lane 5) and by gel filtration chromatography (Fig. 2D). A similar purification of WT gp120-GCN4 trimer proteins was described previously (32).

To further characterize the proteins, we performed ELISA analysis with selected conformational ligands directed against the CD4 binding site. As described above, the original purpose of introducing S375W and T257S mutations was twofold: to enhance the preformation of the gp120 structure that is induced by CD4 binding and to eliminate recognition by CD4BS antibodies. Therefore, we tested the recognition of the potently neutralizing CD4BS antibody b12 and two nonneutralizing CD4BS antibodies, F105 and b6. Upon gp120 binding, the nonneutralizing CD4BS antibodies induce a conformational change to the envelope glycoprotein distinct from that induced by CD4 (47). We have hypothesized that the decreased binding of the CD4BS antibodies as the result of filling the cavity in a manner that favors the CD4-bound conformation occurs as a consequence of inducing a gp120 conformation that is incompatible with CD4BS antibody recognition. However, since the neutralizing CD4BS antibody b12 induces limited, if any, conformational changes to bind gp120 (21, 47), we hypothesized that the stabilization of gp120 in the CD4-bound conformation might have less effect on b12 recognition. In terms of immunogen design, it would be desirable to retain both the b12 epitope and the CD4 binding site in a candidate immunogen engineered to elicit broadly neutralizing antibodies against this region in primary isolates. To test the effects of the mutations, we performed ELISA studies to determine the effect of double mutations on the binding of three CD4BS monoclonal antibodies to envelope proteins. As described in Materials and Methods, WT and mutant affinity-purified proteins were added at equivalent concentrations to lectin-coated ELISA plates, incubated with fivefold-serially-diluted concentrations of the three CD4BS antibodies, and then reacted with HRP-conjugated anti-human IgG for colorimet-

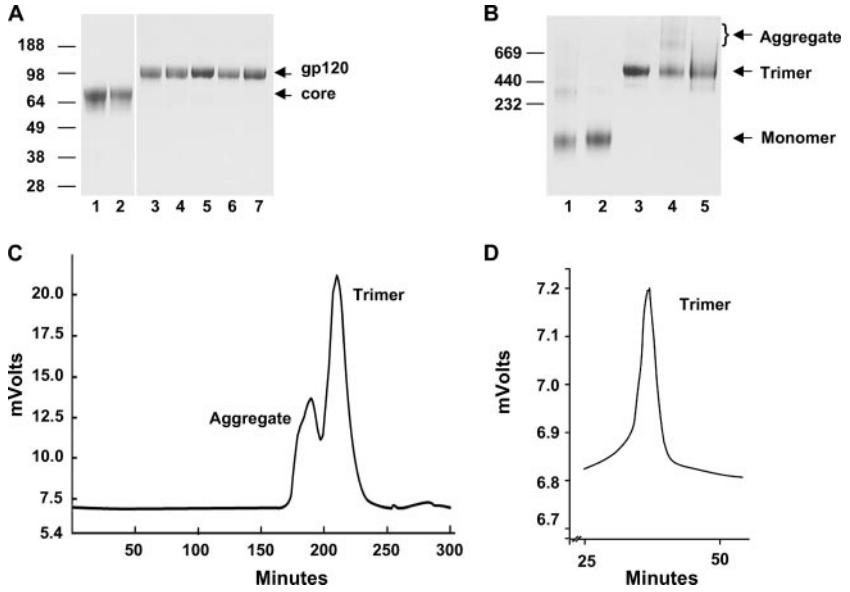


FIG. 2. Reducing SDS-PAGE, blue native gels, and gel filtration profiles of WT and mutant (S375S+T257S) YU2 envelope proteins. (A) Reducing SDS-PAGE of purified envelope glycoproteins. Lane 1, unmodified core; lane 2, mutant core; lane 3, WT gp120; lane 4, mutant gp120; lane 5, WT gp120 trimer purified by gel filtration; lane 6, mutant gp120 trimer before gel filtration; lane 7, mutant gp120 trimer after gel filtration. Molecular weight markers (in thousands) are shown on the left. (B) Blue native gel. Lane 1, WT gp120 monomer; lane 2, mutant gp120 monomer; lane 3, WT trimer purified by gel filtration; lane 4, mutant trimer before gel filtration; lane 5, mutant trimer after gel filtration. Molecular weight markers (in thousands) are indicated on the left. (C) Gel filtration profile of the affinity-purified mutant trimer. (D) Gel filtration profile of the purified mutant trimeric fraction.

ric detection (Fig. 3). Although by immunoprecipitation, the single S375W mutation adversely affected b12 recognition, as shown in Fig. 3, b12 binding to the cavity-filled core proteins was roughly equivalent to unmodified core recognition. In the context of full-length monomeric gp120, b12 recognition of proteins possessing the cavity-filling mutations was detected but was slightly less efficient than that of WT gp120 (Fig. 3). Importantly, the gp120-GCN4 CD4-state double mutant proteins retained a level of b12 binding equivalent to that of the WT trimers (Fig. 3). In contrast, all mutant proteins lost the capacity to be recognized by the nonneutralizing CD4BS antibody F105 (Fig. 3, middle panel), consistent with our previous analysis of the single cavity-altering mutant (47). However, the double cavity-altering mutations did not eliminate recognition by all types of nonneutralizing CD4BS antibodies, as the weakly neutralizing CD4BS antibody b6 remained unaffected by the cavity mutations in all protein contexts (Fig. 3, bottom panel). Thereby, binding levels observed with b6, along with HIV Ig (not shown), served as an internal control for protein equivalence in the assay.

Because b12 recognition of the T257+S375W double mutant proteins was relatively efficient in this analysis, and because the poor recognition of the S375W single mutant was determined previously only by immunoprecipitation, we compared the relative recognition of b12 to the WT, the single mutant, and the double mutant gp120 monomers by ELISA. As seen in Fig. 3B, the single mutant is substantially less well recognized by b12 than is WT gp120. However, the monomeric double mutant gp120 is slightly more efficiently recognized by b12 than the single mutant protein at position 375 (Fig. 3B). In the trimeric gp120 context, the double mutant protein is recognized by b12 equivalently to unmodified gp120-GCN4 glycoproteins.

Effect of the cavity mutations on viral entry and sensitivity to the neutralizing antibody b12. Previously, we observed that the S375W mutation in YU2 Env decreased entry of the pseudotyped virus (47). Since the introduction of a second cavity mutation, T257S, enhanced both protein solubility (Zhou et al., personal communication) and b12 recognition of the double mutant protein relative to the single mutant gp120 monomer at position 375 (Fig. 3), we then asked if the introduction of the mutation at position 257 into full-length Env would impact viral entry or b12 neutralization. As can be seen in Fig. 4A, when we normalized entry to the quantity of p24 in a given volume of the viral stock, the presence of the mutation at position 257 enhanced entry relative to the single mutant envelope glycoprotein at position 375. We sought to determine if any correlation of enhanced b12 recognition of the double mutant monomer could be observed in the context of b12-meditated viral neutralization. Viruses pseudotyped with the YU2 envelope glycoproteins harboring either only the S375W mutation or a combination of S375W and T257S mutations were assessed for entry in the presence of serial dilutions of the b12 antibody. The b12 IC_{50} values showed that viruses harboring the S375W single mutation were approximately threefold less sensitive to antibody-mediated neutralization than viruses possessing the double mutations at positions 257 and 375 (Fig. 4B). HIV IgG and the 2F5 antibody were used as comparative controls in the neutralization assay. The trend of increased neutralization sensitivity of double mutant viruses correlates with increased b12 recognition of gp120 proteins containing the double cavity-filling mutations compared to gp120 glycoproteins possessing only the single S375W mutation as determined by ELISA analysis (Fig. 3B).

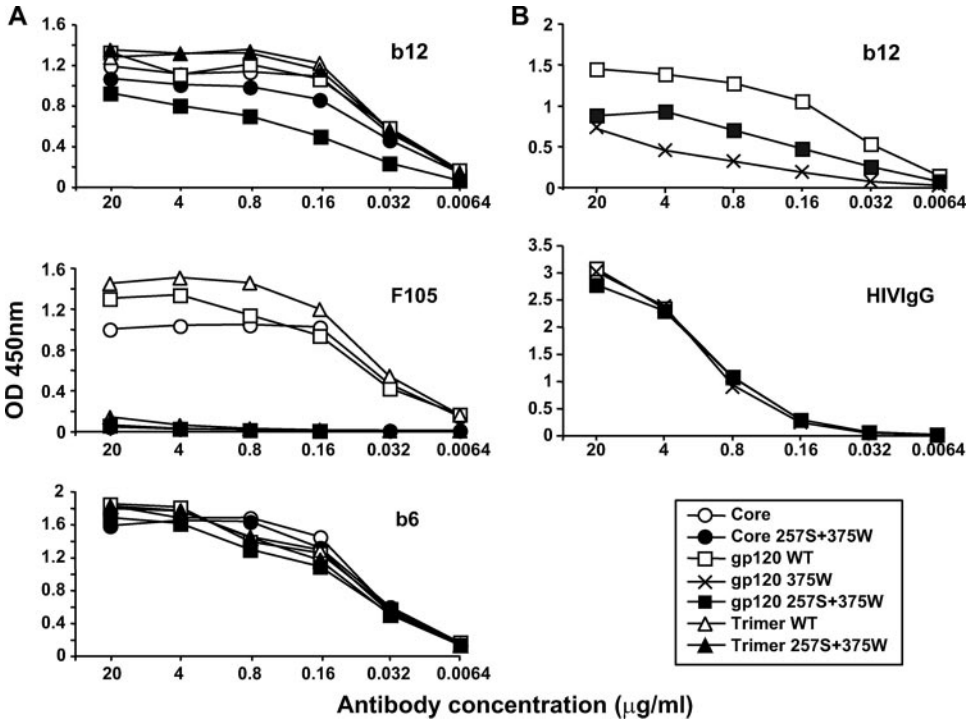
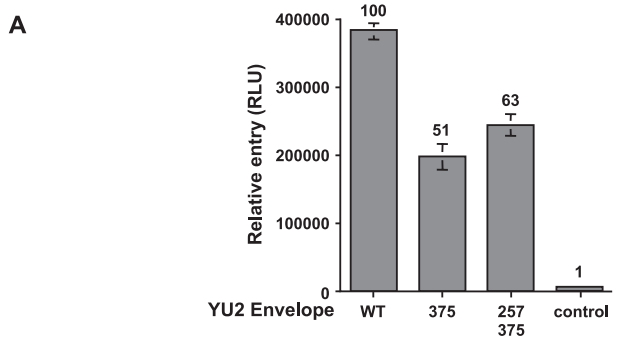


FIG. 3. Antigenicity of WT and mutant glycoproteins. (A) Binding to three CD4 binding-site antibodies was measured by ELISA analysis of the following proteins: WT proteins (open symbols) and T257S+S375W mutant proteins (closed symbols). Top panel, binding to b12; middle panel, binding to F105; bottom panel, binding to b6. (B) Effect of a second-site mutation (T257S) on b12 recognition. Top panel, comparison of binding to b12 by WT gp120 (open square), S375W single mutant gp120 (cross), and T257S+S375W double mutant gp120 (closed square); bottom panel, binding to HIV IgG was used as an internal control for protein concentration. Margins of error from duplicate reactions were negligible in all cases.

Thermodynamic analysis of ligand interactions of mutant envelope glycoproteins. Unmodified proteins and cavity-filled proteins possessing the replacements at positions 257 and 375 were assayed by ITC to determine if either CD4 affinity or changes in entropy induced by CD4 were altered in the mutant glycoproteins. Figure 5 shows the titration profiles for interaction of the WT (left panel) and T257S+S375W mutant (right panel) core proteins with CD4. As mentioned in Materials and



B

	Antibody concentration (μg/ml)								
	HIVIgG			b12			2F5		
Virus	WT	375W	257S+375W	WT	375W	257S+375W	WT	375W	257S+375W
IC_{50}	1088	904	861	1.85	8	3.16	10.3	14.9	10.4

FIG. 4. Entry and b12 neutralization of pseudotyped WT and mutant HIV-1. (A) Entry of HIV-1 pseudotyped with WT, the S375W single mutant, and the T257S+S375W double mutant YU2 Env. Percent relative entries compared to WT virus are indicated in the graph. The average values of four replicates from one representative experiment have been plotted. *y*-axis error bars indicate standard deviations of the replicate values. (B) Neutralization IC_{50} values of the three viruses with selected antibodies are shown.

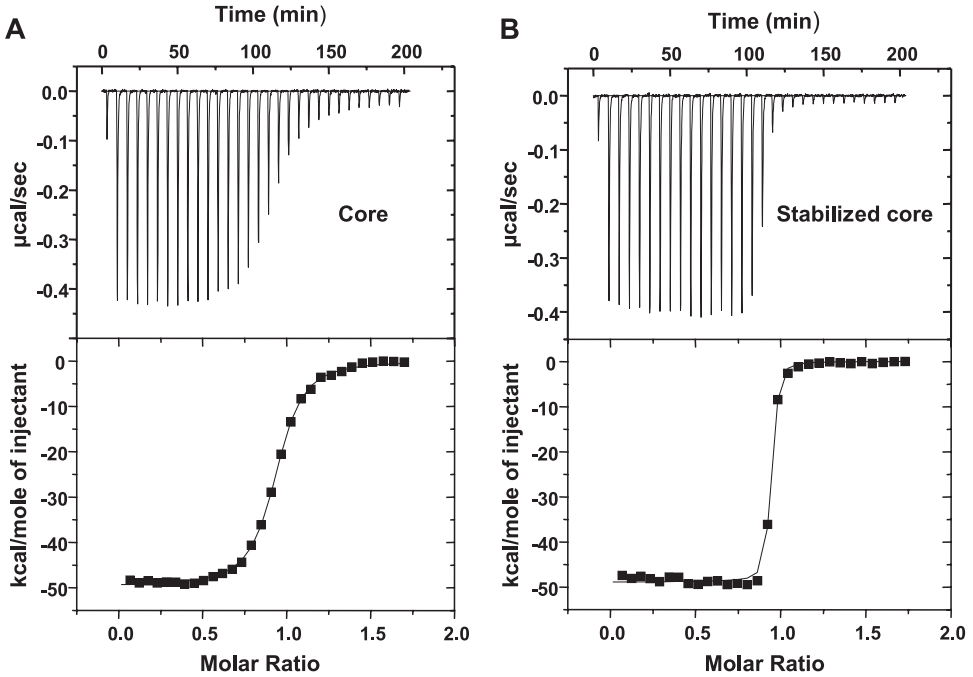


FIG. 5. Isothermal titration calorimetry analyses of unmodified and mutant (T257S+S375W) YU2 core proteins. ITC experiments representing the interactions of sCD4 with unmodified (A) and mutant (B) core proteins at 37°C are shown. The top panels represent the raw data as power versus time. The area under each spike is proportional to the heat produced for each injection. The bottom panels represent integrated areas per mole of injected ligand (sCD4) as a function of the molar ratio. The solid line represents the best nonlinear fit to the experimental data. (A) $\Delta H = -50$ kcal/mol; $K_d = 29$ nM. (B) $\Delta H = -49$ kcal/mol; $K_d = 1$ nM.

Methods, the values for enthalpy (ΔH), entropy (ΔS), and the association constant (K_a) were obtained by fitting the titration data to a nonlinear least-squares analysis. The dissociation constant of CD4 binding to each protein variant was obtained from the inverse of K_a values. The change in enthalpy upon the binding of CD4 to unmodified core protein (−50 kcal/mol) was close to that observed for the mutant core protein (−49 kcal/mol). Unexpectedly, in the core context, the two cavity-filling changes greatly increased the affinity for CD4 (1 nM) compared to that for the WT core protein (29 nM). Similar measurements were performed for WT and double mutant proteins in the context of the gp120 monomer as well as in the context of gp120-GCN4 trimers. Table 2 summarizes the enthalpies, entropies, and affinities of these proteins for CD4 binding. The overall enthalpy is higher for full-length gp120 proteins than for the core, both as monomers and as trimers, consistent with the fact that these proteins contain more residues that are capable of rearrangement and bond formation than does the core. Similar to the core proteins, full-length mutant gp120 showed increased affinity for CD4 binding, changing from 42 nM for the WT protein to 3 nM for the mutant protein. Improved CD4 affinity was also observed for the trimeric mutant, increasing from 58 nM for the WT gp120-GCN4 trimer to 14 nM for the cavity-filled trimer. This increased affinity was associated with a corresponding decrease in entropy in all cases, but the degree of stabilization when the WT was compared to the T257S+S375W versions differed depending upon the protein context. In the core context, stabilization impacted by the cavity-altering mutations was minimal, approximately 8% relative to unmodified core. As monomeric gp120, the mutations at positions 257 and 375 reduced entropy by 25%, and as trimeric gp120, the mutations reduced entropy by approximately 40% (see the $-T\Delta\Delta S$ values in Table 2). These values reflect increased bond formation on the more complete gp120 molecules and also indicate that the protein context may be an important consideration for designing the most stabilized protein immunogen. We conclude that the gp120-GCN4 cavity-altered trimeric glycoproteins are partially

TABLE 2. Thermodynamic values of YU2 gp120 variants and sCD4 interactions at 37°C measured by ITC

Protein	ΔG (kcal/mol)	ΔH (kcal/mol)	$-T\Delta S$ (kcal/mol)	K_d (nM)	$-T\Delta\Delta S$ (kcal/mol)[a]
WT core	−10.7 ± 0.04	−49.6 ± 2.48	38.9 ± 2.48	29	
Stabilized core	−12.7 ± 0.12	−48.8 ± 2.45	36.0 ± 2.45	1	2.9
WT gp120	−10.5 ± 0.05	−68.8 ± 3.46	58.3 ± 3.46	42	
Stabilized gp120	−12.0 ± 0.09	−56.1 ± 2.82	44.0 ± 2.82	3	14.0
WT trimer	−10.3 ± 0.06	−64.2 ± 3.27	53.9 ± 3.27	58	
Stabilized trimer	−11.1 ± 0.08	−44.5 ± 2.25	33.4 ± 2.25	14	20.4

[a] The $-T\Delta\Delta S$ values were calculated by subtracting the $-T\Delta S$ value of each mutant from that of its unmodified counterpart.

TABLE 3. Endpoint titers of sera from rabbits immunized at low and high doses of glycoproteins as determined by gp120 ELISA[a]

Immunogen	Inoculation					
	Low dose		High dose			
	Animal	Endpoint titer (3rd)	Animal	Endpoint titer		
				2nd	3rd	4th
WT monomer	A1	+++++	3	+++++	+++++	++++
	A2	++++	4	+++	++++	+++++
	A3	++++	5	++++	+++++	++++
			6	+++++	++++	+++++
WT trimer	B1	++++	7	+++++	+++++	++++
	B2	++++	8	ND[b]	+++++	+++++
	B3	++++	9	+++++	++++	++++
			10	+++++	+++++	++++
Stabilized trimer	C1	++++	11	+++++	++++	+++++
	C2	++++	12	+++++	+++++	+++++
	C3	++++	13	+++++	+++++	+++++
			14	++++	+++	++++
			15	+++++	++++	+++
			16	+++++	+++++	+++++
			17	+++++	+++++	++++

[a] Endpoint ELISA titers are defined as the last reciprocal serum dilution at which the optical density signal was greater than twofold over the signal detected with the preimmune sera. Symbols for the endpoint titers are as follows: +++, 2.5×10^4; ++++, 1.25×10^5; +++++, 6.25×10^5.
[b] ND, not determined.

stabilized in the CD4-bound state, as previously demonstrated for monomeric gp120 possessing the single S/W mutation at position 375 (47). These observations supported a rationale to assess the stabilized double mutant proteins for immunogenicity analysis.

Comparative immunogenicity of the gp120 variants in rabbits. In this study, gp120-GCN4 double mutant proteins displayed low nanomolar binding to CD4, exhibited b12 recognition equivalent to that of WT gp120, and demonstrated the largest change in entropy upon CD4 binding compared to those of other mutant variants as determined by ITC. Therefore, we selected this CD4-state mimetic as our best candidate to assess the impact of the cavity-altering mutations on immunogenicity. Assessments of the antibody responses elicited in rabbits by affinity-purified WT gp120 (four animals), WT gp120-GCN4 (four animals), and T257S+S375W gp120-GCN4 proteins (seven animals) were performed at two doses: at the 25-μg dose used in a previous study (24) and at a higher dose of 125 μg of protein to determine if this quantity of protein would elicit more potent and broad responses. All proteins were emulsified in Ribi adjuvant and inoculated at 4-week intervals as described in Materials and Methods. Test bleeds were collected 10 days following each inoculation, and the isolated sera were subjected to ELISA and HIV-1 neutralization assays. Preimmune bleeds were analyzed similarly, and two rabbits inoculated with BSA in adjuvant served as additional negative controls. We also included viruses pseudotyped with murine leukemia virus Env to confirm that the neutralization was specific for HIV Env.

The sera from animals inoculated with both low and high doses of protein were collected and tested for binding activity to WT YU2 gp120 protein by ELISA; the results are summarized in Table 3. All of the animals inoculated with the gp120 variants achieved peak titers of anti-gp120 IgG after two to three inoculations, with the endpoint titers ranging from 2.5×10^4 to 6.25×10^5. Sera from the low-dose-inoculated animals were tested after three inoculations only and had roughly the same titer as the sera from high-dose-inoculated animals. Sera from BSA-immunized rabbits had no detectable titer for gp120-reactive antibodies (data not shown).

Next, we examined the breadth of HIV neutralization elicited by each of the immunogens after three inoculations for the both low-dose- and high-dose-inoculated animals. As seen in Fig. 6, sera derived from animals inoculated with the higher dose of protein were much more potent at neutralizing a panel of viruses than sera from those inoculated at the lower dose. Therefore, we performed another inoculation of the animals receiving the high dose only.

Even though there were not large differences in the ELISA titers, the sera elicited by either WT or partially stabilized proteins following the fourth inoculation displayed more potent neutralization than the sera isolated following the third inoculation (Fig. 6). In general, the breadth of neutralization improved after four inoculations, with IC_{50} values greater than 10 observable in most sera for seven of the nine primary clade B isolates that were tested. Due to the small number of animals per group, the ability to perform statistical analysis was limited. However, by combining all immunogens against all neutralized viruses, a statistically significant difference between the IC_{50} values after three inoculations compared to those following four inoculations was observed by the evaluation of the arithmetic means by an unpaired t test ($P = 0.003$). By inspection and statistical analysis of the IC_{50} neutralization values following four inoculations, there was no apparent advantage in YU2 homologous neutralization elicited by WT trimeric gp120 glycoproteins compared to that elicited by WT monomeric gp120 proteins (Fig. 6). Therefore, we combined the IC_{50}

Immunogen	Low dose ID#	High dose ID#	YU2			SF162			BaL.01			SS1196			6535		
			Low	High		Low	High		Low	High		Low	High		Low	High	
			Post-3	Post-3	Post-4	Post-3	Post-3	Post-4	Post-3	Post-3	Post-4	Post-3	Post-3	Post-4	Post-3	Post-3	Post-4
BSA		1			<10			<10			<10			<10			<10
		2			<10			<10			<10			<10			<10
WT monomer	A1	3	<10	422	563	13	10	141	<10	38	61	<10	12	177	<10	<10	84
	A2	4	<10	85	53	<10	31	242	<10	24	10	<10	<10	166	<10	<10	65
	A3	5	16	26	34	<10	74	88	<10	60	<10	<10	21	21	<10	56	<10
		6		13	7		<10	14		<10	<10		<10	15		<10	<10
WT trimer	C1	7	<10	42	49	58	26	194	<10	45	112	14	15	184	<10	24	361
	C2	8	<10	32	112	<10	40	425	<10	38	86	<10	13	530	<10	<10	597
	C3	9	<10	269	59	<10	44	16	<10	<10	<10	<10	<10	32	<10	<10	19
		10		35	23		<10	16		<10	10		<10	83		<10	<10
Stabilized trimer	D1	11	120	167	274	<10	12	741	<10	40	474	<10	24	1138	<10	<10	390
	D2	12	87	219	2387	49	28	527	<10	168	236	<10	181	2939	<10	72	243
	D3	13	<10	30	81	<10	33	499	<10	17	22	<10	21	18	<10	36	53
		14		<10	15		<10	15		<10	<10		<10	41		<10	<10
		15		1132	1909		<10	187		50	10		<10	<10		<10	<10
		16		664	2190		61	320		177	573		62	827		181	62
		17		31	25		84	260		45	563		28	319		30	29

Immunogen	Low dose ID#	High dose ID#	ADA			89.6			JRFL			TRJO.58			MuLV.SG3		
			Low	High		Low	High		Low	High		Low	High		Low	High	
			Post-3	Post-3	Post-4	Post-3	Post-3	Post-4	Post-3	Post-3	Post-4	Post-3	Post-3	Post-4	Post-3	Post-3	Post-4
BSA		1			<10		<10	<10		<10	<10		<10	<10			<10
		2			<10		<10	<10		<10	<10		<10	<10			10
WT monomer	A1	3	23	41	199	<10	<10	<10	<10	<10	12	<10	<10	<10	<10	<10	<10
	A2	4	<10	<10	101	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
	A3	5	19	22	23	<10	<10	<10	<10	<10	<10	<10	13	<10	19	<10	<10
		6		14	19		<10	<10		<10	<10		<10	<10		<10	<10
WT trimer	C1	7	<10	33	148	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	17	<10
	C2	8	<10	<10	207	<10	<10	206	<10	<10	13	<10	<10	<10	<10	<10	<10
	C3	9	<10	<10	29	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
		10		24	66		<10	18		<10	<10		<10	<10		<10	<10
Stabilized trimer	D1	11	<10	36	71	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	12	<10
	D2	12	<10	155	325	<10	<10	165	<10	<10	<10	<10	<10	<10	<10	<10	<10
	D3	13	<10	60	47	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	19	<10
		14		<10	38		<10	17		<10	<10		<10	<10		<10	<10
		15		<10	<10		<10	<10		<10	<10		<10	<10		<10	<10
		16		156	335		<10	239		<10	<10		<10	<10		<10	<10
		17		<10	182		<10	127		<10	<10		<10	<10		<10	<10

FIG. 6. IC_{50} neutralization values of different groups of immunized rabbit sera tested against a panel of HIV-1 isolates. Low-dose values were obtained with sera collected after three inoculations (post-3), and high-dose values were obtained with sera collected after three inoculations (post-3) as well as after four inoculations (post-4). IC_{50} values between 100 and 249 are coded in yellow, and those exceeding 250 are highlighted in red. Virus pseudotyped with murine leukemia virus (MuLV) was used as a negative control for specificity.

values for sera elicited by either WT monomeric or WT trimeric gp120 and performed a limited statistical analysis. In contrast to WT gp120-elicited sera, the sera elicited by the T257S+S375W-stabilized trimers displayed much more potent homologous neutralization in several individual animals (Fig. 6). A comparison of the arithmetic mean IC_{50} neutralization values between the two groups is shown in Table 4, and the difference in the arithmetic means for YU2 neutralization was just within the range of statistical significance.

For heterologous neutralization, the stabilized trimers elicited responses against strains BaL, SF162, and SS1196 that by inspection appeared more potent than those elicited by the

TABLE 4. Mean IC_{50} neutralization values of WT proteins compared to those of stabilized trimers

Virus	Mean IC_{50}		P value[c]
	WT gp120[a] (n = 8)[b]	Stabilized trimer (n = 7)	
YU2	121 ± 64	983 ± 421	0.0494
BaL	37 ± 15.3	269 ± 100	0.0287
SF162	142 ± 51	364 ± 91.65	0.0468
SS1196	151 ± 60	755.3 ± 400.2	0.1337
89.6	32 ± 25	80 ± 36.4	0.2798

[a] IC_{50} values obtained with WT gp120 monomer and WT gp120 trimer were combined for the analysis.
[b] n refers to the number of samples (IC_{50} values) considered for analysis.
[c] By unpaired t test. A P value of <0.05 was considered to be significant.

WT proteins. Statistical significance of these differences was observed only for BaL and SF162 neutralization by comparing the arithmetic means of WT gp120-elicited responses to those elicited by the stable trimers (Table 4), and a trend was observed for SS1196. For strains ADA and 6535, both by inspection and by statistical analysis, there was no difference in the IC_{50} values elicited by WT proteins compared to the stabilized trimers. For the resistant viruses JR-FL and TRJO.58, no consistent neutralization was observed, indicating the limits of the neutralization breadth or potency elicited in this study. We did inoculate selected animals two additional times with immunogen, but we observed neither a consistent pattern of enhanced neutralization nor any increase in ELISA titers with the additional boosts.

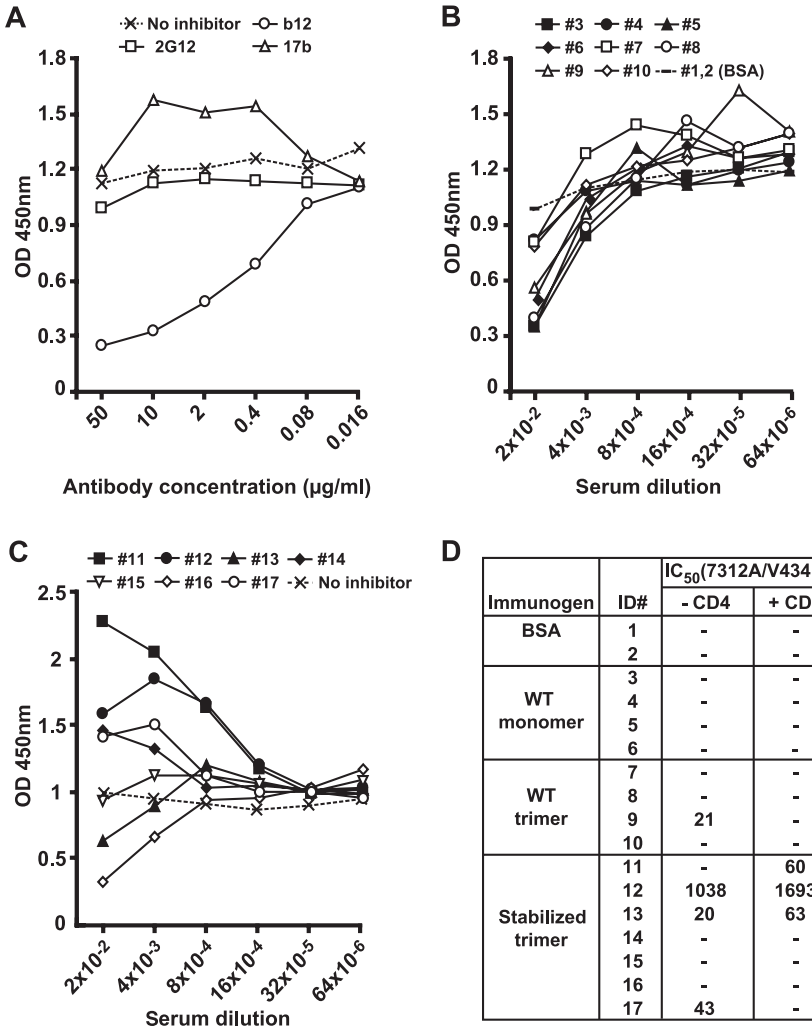
To confirm that the neutralization was mediated by IgG, we treated selected sera with protein A-Sepharose beads and demonstrated that all YU2- and SF162-neutralizing activity could be depleted from these sera by the beads (data not shown). We were also able to elute a substantial fraction of the neutralizing activity from the protein A beads following treatment with acidic buffer and immediate neutralization of the eluates (data not shown). Analysis of the eluates by reducing SDS-PAGE confirmed the presence of bands consistent with the heavy and light chains of purified IgG (data not shown).

In summary, using an Env-pseudotyped HIV neutralization assay, we have shown that at high protein doses, monomeric gp120 elicits relatively broad neutralizing activity and that the cavity-altered, CD4-state gp120 trimers elicit neutralizing antibodies that are slightly more potent than those elicited by the WT monomers and trimers.

Mapping binding and neutralization specificity. Several assays were performed in attempts to identify the subsets of either the binding or neutralizing specificities present in the gp120-elicited antisera. In a recent immunogenicity study using guinea pigs, we observed that most of the homologous neutralization elicited by the YU2 monomeric gp120 in the adjuvant AS01b mapped to a single 15-amino-acid region of the V1 loop of gp120 (24). We performed similar peptide inhibition studies of selected antisera elicited in the rabbits by the high-dose YU2 monomers and trimers emulsified in Ribi adjuvant generated in the current study. Surprisingly, no detectable homologous neutralization elicited by the YU2 gp120 monomers used in this study could be inhibited by the V1 peptide identified in the previous study. Sera displaying the highest titers were analyzed in this manner, two for monomeric gp120 (samples 3 and 4) as well as two antisera elicited by the WT gp120 trimers (samples 7 and 8) and all sera elicited by the stabilized trimers with the exception of sample 31. Additionally, neutralization of YU2 was not V3 mediated, as a YU2 V3 peptide was unable to significantly inhibit the neutralization of selected sera (samples 3, 10, 11, 32, and 33). A minor fraction of neutralizing activity against BaL could be inhibited by the V3 peptide for selected sera (samples 7, 8, 11, 12, 15, and 17) (not shown), and no V3-directed neutralization of ADA was detected (samples 3, 7, 8, 12, 15, and 16).

Because the CD4-state immunogens might elicit a repertoire of antibodies against the CD4 binding region different from that of WT immunogens, we developed novel assays to determine if we could observe differences regarding the inhibition of CD4 binding to gp120 or, alternatively, direct binding of antisera to selected gp120 core variants. Because a previous report described cross-competition effects by monoclonal antibodies that do not map to the CD4 binding region (28), we used YU2 core gp120 as the binding target to eliminate cross-competition from antibodies in the sera that would not have specificity for the CD4 binding region as much as possible (5). In addition, since the gp120 core protein has a deletion of the major variable loops and residues in the N and C termini, a large fraction of the native binding surface is accounted for by the CD4 binding site (22, 45, 46). Therefore, we analyzed the ability of sera elicited by WT proteins to affect the binding of sCD4 to unmodified YU2 core proteins and compared it to that elicited by the constrained proteins (Fig. 7). As assay controls, we preincubated three well-defined antibodies, b12 (CD4BS), 17b (CD4i), and 2G12 (glycan-dependent outer domain), with core gp120 on the ELISA plate and then determined their impact on CD4 binding to the core. As shown in Fig. 7A, the CD4BS antibody b12 can efficiently block CD4 binding to the gp120 core; this interaction is unaffected by 2G12, and interestingly, CD4 binding to the core is enhanced by the preaddition of 17b. Analysis of the experimental sera from the WT protein-immunized rabbits displayed various capacities to inhibit the binding of CD4 to the gp120 core (Fig. 7B). A markedly different spectrum of responses was observed when the sera from the stabilized trimer-immunized rabbits were analyzed similarly (Fig. 7C). Two of the sera (samples 13 and 16) displayed slightly reduced CD4 binding, serum sample 15 had little observable effect, and all other sera (samples 11, 12, 14, and 17) enhanced CD4 binding to the gp120 core. Since the data suggested that there might be 17b-like antibodies present in the sera, we performed assays of neutralization of HIV-2 isolate 7312A/V434M by these sera with or without preincubation of the virus with sCD4 in an assay that is diagnostic for 17b-like antibodies (12). HIV-1 neutralization in the presence of CD4 was observed only in sera elicited with the CD4-state trimers (samples 11 to 13) (Fig. 7D). Of these sera, sample 12 showed a particularly high neutralization response even in the absence of sCD4. Although it is difficult to compare the effects observed in ELISA to those observed in virus neutralization assays, it is clear that the stabilized trimer proteins elicited different types of antibody responses compared to those of the WT immunogens.

We next performed assays of direct binding of the sera to the WT YU2 core and to a recently described, cysteine-constrained core (Ds12F123) derived from the HXBc2 envelope glycoprotein that is recognized by CD4 and b12 but not most other nonneutralizing CD4 binding-site antibodies (49). We reasoned that if the stabilized trimers elicited CD4 binding-site antibodies that possessed binding properties similar to those of CD4 or b12, the sera elicited by these molecules might bind more efficiently to the cysteine-constrained core. As seen in Fig. 8, even though the sera elicited by the stabilized trimers did not recognize the WT YU2 core quite as well as sera elicited by the unmodified proteins (A and C), several sera did recognize the cysteine-constrained HXBc2 core proteins more efficiently (B and D).



Immunogen	ID#	IC_{50}(7312A/V434M) - CD4	IC_{50}(7312A/V434M) + CD4
BSA	1	-	-
	2	-	-
WT monomer	3	-	-
	4	-	-
	5	-	-
	6	-	-
WT trimer	7	-	-
	8	-	-
	9	21	-
	10	-	-
Stabilized trimer	11	-	60
	12	1038	1693
	13	20	63
	14	-	-
	15	-	-
	16	-	-
	17	43	-

FIG. 7. Mapping of gp120 and stabilized trimer-elicited sera tested for the capacity to affect sCD4 binding and for the presence of CD4i antibodies. (A, B, and C) Binding of sCD4 to YU2 core protein was measured by ELISA analysis in the presence of fivefold serial dilutions of the following reagents: the human anti-gp120 antibodies b12, 2G12, and 17b (A); rabbit antisera obtained after four inoculations with WT gp120 proteins (samples 3 to 7) and WT trimer proteins (samples 8 to 11) (B); and antisera obtained after four inoculations with stabilized gp120 trimer protein (C). Each data point is an average of duplicate samples with negligible margins of errors that are not visible on the graphs. Two negative control curves are shown; in A and B, the binding of CD4 in the absence of any inhibitor (marked as no inhibitor) indicates 100% binding; in C, the average values obtained in the presence of BSA-immunized serum samples 1 and 2 are shown. (D) Neutralization IC_{50} values of rabbit sera tested against HIV-2 isolate 7312A/V434M. The pseudovirus was treated with media lacking CD4 (−CD4) or containing 0.5 μg/ml of sCD4 (+CD4) for 1 h prior to adding antisera.

DISCUSSION

The HIV-1 primary receptor-binding site presents a unique challenge with regard to targeting this surface by neutralizing antibodies. Due to the extreme variability of many elements of HIV gp120, this conserved region represents one of the two functionally conserved surfaces on the protein. Since it was shown previously that the second conserved gp120 surface, the chemokine receptor binding site, is not accessible to most neutralizing antibodies, the CD4 binding site represents the best surface to target for the generation of broad neutralizing antibodies. We have attempted a novel means of immunogen design by stabilizing this site on gp120 by structure-guided mutagenesis. We have filled the Phe43 cavity, which lies at the epicenter of the CD4 binding region of gp120, by a selected pair of mutations within the cavity. In this study, we show that similar to a single mutation in the Phe43 cavity of gp120, the paired mutations maintain the increased affinity of gp120 for CD4 in the core context as well as in full-length and trimeric gp120 protein contexts. Each of the cavity-filled proteins demonstrates substantial entropic changes upon CD4 ligation consistent with only partial stabilization of these mutants in the CD4-bound conformation. Importantly, the second-site mutation T257S increases the recognition of the cavity-filled protein by the potent and broadly neutralizing CD4BS antibody b12. Since the double mutant core protein remains dependent on CD4 induction for recognition by 17b (Fig. 1), it is likely that the cavity-filling mutations do not facilitate the formation of the bridging sheet structure. Consistent with this, there is a

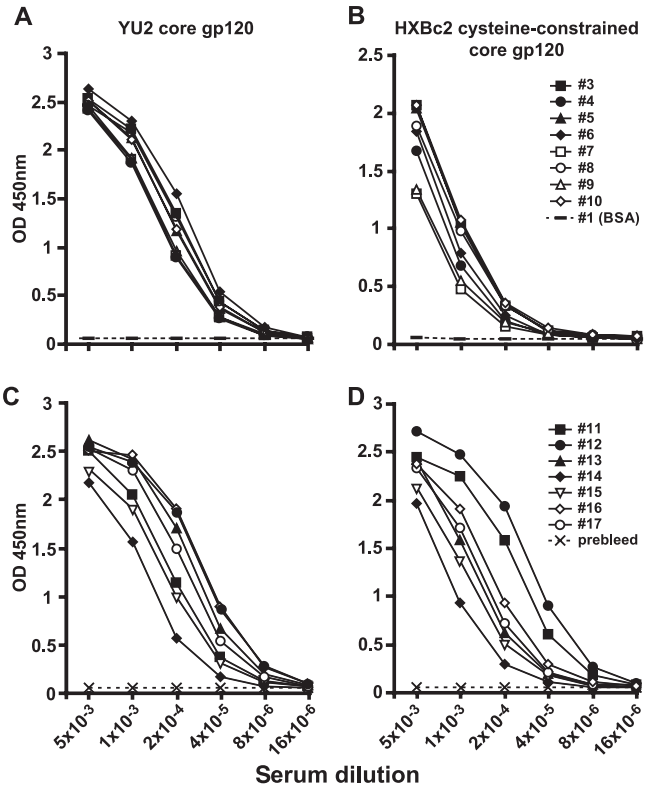


FIG. 8. Binding of WT gp120 and stabilized trimer-elicited sera to unmodified YU2 core and cysteine-constrained HXBc2 core proteins detected by ELISA. (A and C) Binding to YU2 core proteins by antisera elicited by WT gp120 proteins (A) and sera elicited by the stabilized trimers (C). (B and D) Binding to the cysteine-constrained HXBc2 core protein Ds12F123 by antisera elicited by WT gp120 proteins (B) and sera elicited by the stabilized trimers (D). Prebleed antisera and antisera from rabbits inoculated with BSA are included as negative controls.

modest reduction in the entropy change observed during gp120 and CD4 interactions, indicating that the flexibility of gp120 resides in areas other than the CD4 binding region (Table 2). This observation is consistent with differences between the structures of unliganded and liganded core gp120 proteins that suggest that the core inner domain undergoes major structural rearrangements upon binding to CD4 (7, 22). The impact of the T257S+S375W cavity-filling mutations on the change in entropy is greater when the mutations are examined in the full-length gp120 monomer and trimer contexts compared to the core. This difference is likely due to rearrangements of residues in the amino and carboxy termini that are not present in the core, although the variable loop V1, V2, or V3 could contribute to this difference.

Irrespective of the protein context, a mutation at residue 375 disrupts the binding surface for the CD4BS antibody F105 (47), and the T257S+S375W double mutation decreases F105 recognition to an undetectable level. However, the two cavity-filling mutations had no effect on the binding of another CD4BS antibody, b6, and a minimal reduction of the binding of the neutralizing CD4BS antibody b12 (Fig. 3). The finding that b6 binding is not affected is consistent with the mutagenic analysis of b6 and b12, demonstrating that the binding requirements of the nonneutralizing and neutralizing CD4BS antibodies are remarkably similar (33). The data are consistent with the model of conformational masking, which suggests that although CD4 and most CD4BS antibodies bind to a similar region on gp120, they recognize distinct conformations that are incompatible with mutual recognition (47). Since b12 does not induce appreciable conformational changes in gp120 upon binding, it is possible that b12 can recognize the CD4-bound conformation to a certain degree, and the cross-competition observed between these two ligands is predominantly steric (28).

By ELISA, we show that the S375W single mutation decreases gp120 recognition by b12, consistent with data from previous studies. However, for the purposes of immunogen design, we would like to maintain the b12 binding site to permit the elicitation of b12-like antibodies by our candidate immunogens. Therefore, it is significant that the second mutation in the Phe43 cavity at position 257 permits more efficient b12 recognition. The presence of the b12 binding site seems to be a likely requirement for any immunogen that might re-elicit a b12-like antibody. Consistent with the binding analysis, YU2 pseudoviruses harboring the double mutations in Env enter cells more efficiently than those possessing the single mutation. The double mutant viruses also become more sensitive to b12-mediated neutralization than the S375W single mutant.

The double mutant gp120 trimers were tested as immunogens compared to WT gp120 monomer and trimer proteins. Used at higher dosages than those used in previous studies (24), the gp120 monomer itself elicited neutralizing responses against a panel of clade B viruses. Of particular interest was the neutralization of the primary strain ADA (Fig. 5). It is possible that affinity selection and the relatively high functional activity of the YU2 gp120 used in this study contributed to the relatively potent neutralization elicited by the monomer at the higher dose of 125 μg. We have observed that gp120 proteins from tissue culture supernatants purified by antibody affinity chromatography, compared to the purification of the same protein from supernatants by lectin affinity chromatography, display up to threefold-more purified gp120 molecules capable of binding CD4, as determined by ITC (not shown). Such differences in gp120 "functional activity," defined by the capacity to interact with CD4, may be an important but underappreciated variable in immunogenicity experiments involving gp120 expressed from DNA or via viral vectors in vivo or in vitro followed by lectin affinity purification only.

Trimerization of full-length gp120 did not improve the potency or breadth of the neutralizing response. However, the cavity-filled trimers did elicit more potent neutralizing responses against many of the viruses tested, as can be seen by an inspection of Fig. 6. A minimal, if any, increase in the breadth of neutralization was elicited by the partially stabilized trimers. These data indicate that in and of themselves, the cavity-filling mutations examined in the contexts here are not sufficient to increase neutralization breadth.

As an attempt to map the neutralizing activity elicited by the selected immunogens, we performed assays that we have used previously, namely, assays of neutralization competition by variable loop peptides (24), assays to identify CD4i antibodies (12), and novel cross-competition assays to determine the effects of antibodies on the binding of sCD4 to gp120. Recently,

we reported that in guinea pigs, monomeric YU2 gp120 identical to the protein used in the current study, emulsified in the GlaxoSmithKline adjuvant AS01b, elicited a predominant V1-specific homologous neutralizing response. Surprisingly, in the current study, we did not observe such a focused neutralizing response elicited by the YU2 gp120. We can attribute the discrepancy only to differences in the animal species, adjuvant, and dosages used in the two studies. Little V3-directed neutralizing activity was detected, in agreement with our previous analysis. We attempted to determine if there was evidence of increased levels of antibodies directed against the gp120 CD4 binding site, since the immunologic objective desired by stabilizing the gp120 CD4 state is to elicit, in essence, "CD4-like" antibodies. Few assays are ideally suited to identify such antibodies, but by the best assay currently available, we analyzed the ability of the sera elicited by the selected immunogens to competitively inhibit the binding of sCD4 to core gp120. We chose core gp120 because we showed previously that fewer cross-competition effects are manifest on core gp120 devoid of the variable loops than on full-length gp120 (5). Somewhat surprisingly, we could detect some CD4-blocking antibodies elicited by WT gp120, in agreement with a previous study (37). However, in some sera elicited by the CD4-stabilized immunogens, we detected antibodies that were more consistent with the CD4i antibody class both by binding analysis and by neutralization of a modified HIV-2 virus capable of detecting CD4i-type antibodies (Fig. 7). This does not mean that the CD4-state immunogens did not induce CD4-blocking antibodies, since their presence could be masked by the CD4i class. The results clearly demonstrate that the stabilized immunogens do elicit at least one subset of antibodies different from the unmodified immunogens. We observed differences in the antisera elicited by WT proteins compared to those elicited by the stabilized trimers in terms of recognition of the recently described cysteine-constrained HXBc2 core proteins (49). These differences might be due to the slightly more efficient elicitation of CD4BS or CD4i antibodies by the CD4-state trimers. Such differences, although not fully defined, likely contribute to the slightly more potent neutralizing antibodies elicited by the stabilized trimeric proteins.

Since we have recently reported that gp140 trimers do elicit enhanced neutralization breadth, it is possible that these partially stabilizing mutations, in concert with other modifications, may present a path to enhance the neutralizing responses elicited by the soluble and stable gp140 trimers further (24). In addition, we plan to stabilize gp120 in the CD4-bound conformation further by altering suitable pairs of amino acids to cysteine residues, especially pairs that would span the inner and outer domains of gp120. Towards that goal, a series of disulfide-stabilized mutants have been generated and will be evaluated in future immunological studies.

ACKNOWLEDGMENTS

We thank Tongqing Zhou for sharing unpublished data regarding solubility of core proteins. We thank Gary Nabel for helpful discussions, Arne Schon for guidance regarding ITC analysis, and Brenda Hartman for assistance with the preparation of the figures.

This study was supported by the Intramural Research Program of the Vaccine Research Center, NIAID, NIH, by the International AIDS Vaccine Initiative, and by a grant from the Bill and Melinda Gates Foundation Grand Challenges of the Global Health Initiative.

REFERENCES

1. **Alkhatib, G., C. Combadiere, C. C. Broder, Y. Feng, P. E. Kennedy, P. M. Murphy, and E. A. Berger.** 1996. CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. Science **272:**1955–1958.
2. **Barnett, S. W., S. Rajasekar, H. Legg, B. Doe, D. H. Fuller, J. R. Haynes, C. M. Walker, and K. S. Steimer.** 1997. Vaccination with HIV-1 gp120 DNA induces immune responses that are boosted by a recombinant gp120 protein subunit. Vaccine **15:**869–873.
3. **Belshe, R. B., G. J. Gorse, M. J. Mulligan, T. G. Evans, M. C. Keefer, J. L. Excler, A. M. Duliege, J. Tartaglia, W. I. Cox, J. McNamara, K. L. Hwang, A. Bradney, D. Montefiori, K. J. Weinhold, et al.** 1998. Induction of immune responses to HIV-1 by canarypox virus (ALVAC) HIV-1 and gp120 SF-2 recombinant vaccines in uninfected volunteers. AIDS **12:**2407–2415.
4. **Berman, P. W., T. J. Gregory, L. Riddle, G. R. Nakamura, M. A. Champe, J. P. Porter, F. M. Wurm, R. D. Hershberg, E. K. Cobb, and J. W. Eichberg.** 1990. Protection of chimpanzees from infection by HIV-1 after vaccination with recombinant glycoprotein gp120 but not gp160. Nature **345:**622–625.
5. **Binley, J. M., R. Wyatt, E. Desjardins, P. D. Kwong, W. Hendrickson, J. P. Moore, and J. Sodroski.** 1998. Analysis of the interaction of antibodies with a conserved enzymatically deglycosylated core of the HIV type 1 envelope glycoprotein 120. AIDS Res. Hum. Retrovir. **14:**191–198.
6. **Burton, D. R., J. Pyati, R. Koduri, S. J. Sharp, G. B. Thornton, P. W. Parren, L. S. Sawyer, R. M. Hendry, N. Dunlop, P. L. Nara, et al.** 1994. Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. Science **266:**1024–1027.
7. **Chen, B., E. M. Vogan, H. Gong, J. J. Skehel, D. C. Wiley, and S. C. Harrison.** 2005. Structure of an unliganded simian immunodeficiency virus gp120 core. Nature **433:**834–841.
8. **Choe, H., M. Farzan, Y. Sun, N. Sullivan, B. Rollins, P. D. Ponath, L. Wu, C. R. Mackay, G. LaRosa, W. Newman, N. Gerard, C. Gerard, and J. Sodroski.** 1996. The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. Cell **85:**1135–1148.
9. **Colman, P. M., and M. C. Lawrence.** 2003. The structural biology of type I viral membrane fusion. Nat. Rev. Mol. Cell Biol. **4:**309–319.
10. **Connor, R. I., B. T. Korber, B. S. Graham, B. H. Hahn, D. D. Ho, B. D. Walker, A. U. Neumann, S. H. Vermund, J. Mestecky, S. Jackson, E. Fenamore, Y. Cao, F. Gao, S. Kalams, K. J. Kunstman, D. McDonald, N. McWilliams, A. Trkola, J. P. Moore, and S. M. Wolinsky.** 1998. Immunological and virological analyses of persons infected by human immunodeficiency virus type 1 while participating in trials of recombinant gp120 subunit vaccines. J. Virol. **72:**1552–1576.
11. **Dalgleish, A. G., P. C. Beverley, P. R. Clapham, D. H. Crawford, M. F. Greaves, and R. A. Weiss.** 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature **312:**763–767.
12. **Decker, J. M., F. Bibollet-Ruche, X. Wei, S. Wang, D. N. Levy, W. Wang, E. Delaporte, M. Peeters, C. A. Derdeyn, S. Allen, E. Hunter, M. S. Saag, J. A. Hoxie, B. H. Hahn, P. D. Kwong, J. E. Robinson, and G. M. Shaw.** 2005. Antigenic conservation and immunogenicity of the HIV coreceptor binding site. J. Exp. Med. **201:**1407–1419.
13. **Deng, H., R. Liu, W. Ellmeier, S. Choe, D. Unutmaz, M. Burkhart, P. Di Marzio, S. Marmon, R. E. Sutton, C. M. Hill, C. B. Davis, S. C. Peiper, T. J. Schall, D. R. Littman, and N. R. Landau.** 1996. Identification of a major co-receptor for primary isolates of HIV-1. Nature **381:**661–666.
14. **Dragic, T., V. Litwin, G. P. Allaway, S. R. Martin, Y. Huang, K. A. Nagashima, C. Cayanan, P. J. Maddon, R. A. Koup, J. P. Moore, and W. A. Paxton.** 1996. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. Nature **381:**667–673.
15. **Earl, P. L., W. Sugiura, D. C. Montefiori, C. C. Broder, S. A. Lee, C. Wild, J. Lifson, and B. Moss.** 2001. Immunogenicity and protective efficacy of oligomeric human immunodeficiency virus type 1 gp140. J. Virol. **75:**645–653.
16. **Feng, Y., C. C. Broder, P. E. Kennedy, and E. A. Berger.** 1996. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science **272:**872–877.
17. **Gallo, S. A., C. M. Finnegan, M. Viard, Y. Raviv, A. Dimitrov, S. S. Rawat, A. Puri, S. Durell, and R. Blumenthal.** 2003. The HIV Env-mediated fusion reaction. Biochim. Biophys. Acta **1614:**36–50.
18. **Garlick, R. L., R. J. Kirschner, F. M. Eckenrode, W. G. Tarpley, and C. S. Tomich.** 1990. Escherichia coli expression, purification, and biological activity of a truncated soluble CD4. AIDS Res. Hum. Retrovir. **6:**465–479.
19. **Grundner, C., Y. Li, M. Louder, J. Mascola, X. Yang, J. Sodroski, and R. Wyatt.** 2005. Analysis of the neutralizing antibody response elicited in rabbits by repeated inoculation with trimeric HIV-1 envelope glycoproteins. Virology **331:**33–46.
20. **Korber, B., F. Foley, C. Kuiken, S. Pillai, and J. Sodroski.** 1998. Numbering positions in HIV relative to HXBc2. Los Alamos National Laboratory, Los Alamos, NM.
21. **Kwong, P. D., M. L. Doyle, D. J. Casper, C. Cicala, S. A. Leavitt, S. Majeed, T. D. Steenbeke, M. Venturi, I. Chaiken, M. Fung, H. Katinger, P. W. Parren, J. Robinson, D. Van Ryk, L. Wang, D. R. Burton, E. Freire, R. Wyatt,**

J. Sodroski, W. A. Hendrickson, and J. Arthos. 2002. HIV-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites. Nature **420:**678–682.
22. **Kwong, P. D., R. Wyatt, J. Robinson, R. W. Sweet, J. Sodroski, and W. A. Hendrickson.** 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature **393:**648–659.
23. **Li, M., F. Gao, J. R. Mascola, L. Stomatatos, V. R. Polonis, M. Koutsoukos, G. Voss, P. Goepfert, P. Gilbert, K. M. Greene, M. Bilska, D. L. Kothe, J. F. Salazar-Gonzalez, X. Wei, J. M. Decker, B. Hahn, and D. Montefiori.** 2005. Human immunodeficiency type 1 *env* clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies. J. Virol. **79:**10108–10125.
24. **Li, Y., K. Svela, N. L. Mathy, G. Voss, J. R. Mascola, and R. Wyatt.** 2006. Characterization of antibody responses elicited by human immunodeficiency virus type 1 primary isolate trimeric and monomeric envelope glycoproteins in selected adjuvants. J. Virol. **80:**1414–1426.
25. **Mascola, J. R., M. K. Louder, C. Winter, R. Prabhakara, S. C. De Rosa, D. C. Douek, B. J. Hill, D. Gabuzda, and M. Roederer.** 2002. Human immunodeficiency virus type 1 neutralization measured by flow cytometric quantitation of single-round infection of primary human T cells. J. Virol. **76:**4810–4821.
26. **Mascola, J. R., S. W. Snyder, O. S. Weislow, S. M. Belay, R. B. Belshe, D. H. Schwartz, M. L. Clements, R. Dolin, B. S. Graham, G. J. Gorse, M. C. Keefer, M. J. McElrath, M. C. Walker, K. F. Wagner, J. G. McNeil, F. E. McCutchan, D. S. Burke, et al.** 1996. Immunization with envelope subunit vaccine products elicits neutralizing antibodies against laboratory-adapted but not primary isolates of human immunodeficiency virus type 1. J. Infect. Dis. **173:**340–348.
27. **McGaughey, G. B., M. Citron, R. C. Danzeisen, R. M. Freidinger, V. M. Garsky, W. M. Hurni, J. G. Joyce, X. Liang, M. Miller, J. Shiver, and M. J. Bogusky.** 2003. HIV-1 vaccine development: constrained peptide immunogens show improved binding to the anti-HIV-1 gp41 MAb. Biochemistry **42:**3214–3223.
28. **Moore, J. P., and J. Sodroski.** 1996. Antibody cross-competition analysis of the human immunodeficiency virus type 1 gp120 exterior envelope glycoprotein. J. Virol. **70:**1863–1872.
29. **Muster, T., F. Steindl, M. Purtscher, A. Trkola, A. Klima, G. Himmler, F. Ruker, and H. Katinger.** 1993. A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. J. Virol. **67:**6642–6647.
30. **Myszka, D. G., R. W. Sweet, P. Hensley, M. Brigham-Burke, P. D. Kwong, W. A. Hendrickson, R. Wyatt, J. Sodroski, and M. L. Doyle.** 2000. Energetics of the HIV gp120-CD4 binding reaction. Proc. Natl. Acad. Sci. USA **97:**9026–9031.
31. **Ofek, G., M. Tang, A. Sambor, H. Katinger, J. R. Mascola, R. Wyatt, and P. D. Kwong.** 2004. Structure and mechanistic analysis of the anti-human immunodeficiency virus type 1 antibody 2F5 in complex with its gp41 epitope. J. Virol. **78:**10724–10737.
32. **Pancera, M., J. Lebowitz, A. Schon, P. Zhu, E. Freire, P. D. Kwong, K. H. Roux, J. Sodroski, and R. Wyatt.** 2005. Soluble mimetics of human immunodeficiency virus type 1 viral spikes produced by replacement of the native trimerization domain with a heterologous trimerization motif: characterization and ligand binding analysis. J Virol. **79:**9954–9969.
33. **Pantophlet, R., E. O. Saphire, P. Poignard, P. W. Parren, I. A. Wilson, and D. R. Burton.** 2003. Fine mapping of the interaction of neutralizing and nonneutralizing monoclonal antibodies with the CD4 binding site of human immunodeficiency virus type 1 gp120. J. Virol. **77:**642–658.
34. **Posner, M. R., L. A. Cavacini, C. L. Emes, J. Power, and R. Byrn.** 1993. Neutralization of HIV-1 by F105, a human monoclonal antibody to the CD4 binding site of gp120. J. Acquir. Immune Defic. Syndr. **6:**7–14.
35. **Posner, M. R., T. Hideshima, T. Cannon, M. Mukherjee, K. H. Mayer, and R. A. Byrn.** 1991. An IgG human monoclonal antibody that reacts with HIV-1/GP120, inhibits virus binding to cells, and neutralizes infection. J. Immunol. **146:**4325–4332.
36. **Purtscher, M., A. Trkola, A. Grassauer, P. M. Schulz, A. Klima, S. Dopper, G. Gruber, A. Buchacher, T. Muster, and H. Katinger.** 1996. Restricted antigenic variability of the epitope recognized by the neutralizing gp41 antibody 2F5. AIDS **10:**587–593.
37. **Selvarajah, S., B. Puffer, R. Pantophlet, M. Law, R. W. Doms, and D. R. Burton.** 2005. Comparing antigenicity and immunogenicity of engineered gp120. J. Virol. **79:**12148–12163.
38. **Srivastava, I. K., L. Stamatatos, E. Kan, M. Vajdy, Y. Lian, S. Hilt, L. Martin, C. Vita, P. Zhu, K. H. Roux, L. Vojtech, D. C. Montefiori, J. Donnelly, J. B. Ulmer, and S. W. Barnett.** 2003. Purification, characterization, and immunogenicity of a soluble trimeric envelope protein containing a partial deletion of the V2 loop derived from SF162, an R5-tropic human immunodeficiency virus type 1 isolate. J. Virol. **77:**11244–11259.
39. **Sullivan, N., Y. Sun, J. Li, W. Hofmann, and J. Sodroski.** 1995. Replicative function and neutralization sensitivity of envelope glycoproteins from primary and T-cell line-passaged human immunodeficiency virus type 1 isolates. J. Virol. **69:**4413–4422.
40. **Thali, M., C. Furman, D. D. Ho, J. Robinson, S. Tilley, A. Pinter, and J. Sodroski.** 1992. Discontinuous, conserved neutralization epitopes overlapping the CD4-binding region of human immunodeficiency virus type 1 gp120 envelope glycoprotein. J. Virol. **66:**5635–5641.
41. **Thali, M., J. P. Moore, C. Furman, M. Charles, D. D. Ho, J. Robinson, and J. Sodroski.** 1993. Characterization of conserved human immunodeficiency virus type 1 gp120 neutralization epitopes exposed upon gp120-CD4 binding. J. Virol. **67:**3978–3988.
42. **Thali, M., U. Olshevsky, C. Furman, D. Gabuzda, M. Posner, and J. Sodroski.** 1991. Characterization of a discontinuous human immunodeficiency virus type 1 gp120 epitope recognized by a broadly reactive neutralizing human monoclonal antibody. J. Virol. **65:**6188–6193.
43. **Trkola, A., M. Purtscher, T. Muster, C. Ballaun, A. Buchacher, N. Sullivan, K. Srinivasan, J. Sodroski, J. P. Moore, and H. Katinger.** 1996. Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. J. Virol. **70:**1100–1108.
44. **Wrin, T., T. P. Loh, J. C. Vennari, H. Schuitemaker, and J. H. Nunberg.** 1995. Adaptation to persistent growth in the H9 cell line renders a primary isolate of human immunodeficiency virus type 1 sensitive to neutralization by vaccine sera. J. Virol. **69:**39–48.
45. **Wyatt, R., P. D. Kwong, E. Desjardins, R. W. Sweet, J. Robinson, W. A. Hendrickson, and J. G. Sodroski.** 1998. The antigenic structure of the HIV gp120 envelope glycoprotein. Nature **393:**705–711.
46. **Wyatt, R., and J. Sodroski.** 1998. The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. Science **280:**1884–1888.
47. **Xiang, S. H., P. D. Kwong, R. Gupta, C. D. Rizzuto, D. J. Casper, R. Wyatt, L. Wang, W. A. Hendrickson, M. L. Doyle, and J. Sodroski.** 2002. Mutagenic stabilization and/or disruption of a CD4-bound state reveals distinct conformations of the human immunodeficiency virus type 1 gp120 envelope glycoprotein. J. Virol. **76:**9888–9899.
48. **Yang, X., L. Florin, M. Farzan, P. Kolchinsky, P. D. Kwong, J. Sodroski, and R. Wyatt.** 2000. Modifications that stabilize human immunodeficiency virus envelope glycoprotein trimers in solution. J. Virol. **74:**4746–4754.
49. **Zhou, T., L. Xu, B. Dey, A. J. Hassel, D. Van Ryk, S.-H. Huang, X. Yang, M.-Y. Zhang, M. B. Zwick, J. Arthos, D. R. Burton, D. S. Dimitrov, J. Sodroski, R. Wyatt, G. J. Nabel, and P. D. Kwong.** 2007. Structural definition of a conserved neutralization epitope on HIV-1 gp120. Nature **445:**732–737.