Comparison of Uncleaved and Mature Human Immunodeficiency Virus Membrane Envelope Glycoprotein Trimers

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ABSTRACT The mature envelope glycoprotein (Env) spike on the surfaces of human immunodeficiency virus type 1 (HIV-1)-infected cells and virions is derived from proteolytic cleavage of a trimeric gp160 glycoprotein precursor. In these studies, we compared the conformations of cleaved and uncleaved membrane Envs with truncated cytoplasmic tails to those of stabilized soluble gp140 SOSIP.664 Env trimers. Deletion of the gp41 cytoplasmic tail did not significantly affect the sensitivity of viruses with the HIV-1_{AD8} Env to inhibition by antibodies or a CD4-mimetic compound. After glutaraldehyde fixation and purification from membranes, a cleaved Env exhibited a hydrodynamic radius of ~10 nm and an antibody-binding profile largely consistent with that expected based on virus neutralization sensitivity. The purified cleaved Env trimers exhibited a hollow architecture with a central void near the trimer axis. Uncleaved Env, cross-linked and purified in parallel, exhibited a hydrodynamic radius similar to that of the cleaved Env. However, the uncleaved Env was recognized by poorly neutralizing antibodies and appeared by negative-stain electron microscopy to sample multiple conformations. Compared with membrane Envs, stabilized soluble gp140 SOSIP.664 Env trimers appear to be more compact, as reflected in their smaller hydrodynamic radii and negative-stain electron microscopy structures. The antigenic features of the soluble gp140 SOSIP.664 Env trimers differed from those of the cleaved membrane Env, particularly in gp120 V3 and some CD4-binding-site epitopes. Thus, proteolytic maturation allows the membrane-anchored Env to achieve a conformation that retains functional metastability but masks epitopes for poorly neutralizing antibodies.

IMPORTANCE The entry of human immunodeficiency virus type 1 (HIV-1) into host cells is mediated by the envelope glycoprotein (Env) spike on the surface of the virus. Host antibodies elicited during natural HIV-1 infection or by vaccination can potentially recognize the Env spike and block HIV-1 infection. However, the changing shape of the HIV-1 Env spike protects the virus from antibody binding. Understanding the shapes of natural and man-made preparations of HIV-1 Envs will assist the development of effective vaccines against the virus. Here, we evaluate the effects of several Env modifications commonly used to produce Env preparations for vaccine studies and the determination of structure. We found that the cleavage of the HIV-1 Env precursor helps Env to assume its natural shape, which resists the binding of many commonly elicited antibodies. Stabilized soluble Envs exhibit more compact shapes but expose some Env elements differently than the natural Env.

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The human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein (Env) spike consists of three gp120 exterior subunits and three gp41 transmembrane subunits (1–6). The Env trimer mediates virus entry into host cells by binding the target cell receptors CD4 and CCR5/CXCR4 and fusing the viral and cell membranes (7–13). The HIV-1 Env trimer on virions can potentially sample three conformations, the unliganded, pretriggered conformation (state 1); the full CD4-bound conformation (state 3); and a relaxed, intermediate conformation (state 2) (14, 15). High activation barriers separate the state 1 conformation of most primary HIV-1 Envs from downstream conformations (states 2 and 3) (14–17). CD4 binding lowers these activation barriers and drives Env into states 2 and 3 on the virus entry pathway (14–16).

In the infected cell, the HIV-1 Env trimer is synthesized in the rough endoplasmic reticulum, where signal peptide cleavage, folding, trimerization, and the addition of high-mannose glycans take place (18–20). The resulting gp160 Env precursor is transported to the Golgi apparatus, where proteolytic cleavage by host furin-like proteases produces the gp120 and gp41 subunits (21–27). The mature gp120 and gp41 glycoproteins remain associated through noncovalent bonds (1, 4, 28). The proteolytically processed Env trimers are transported to the cell surface and incorporated into budding virions.

During HIV-1 infection, antibodies are elicited against Env. Recognition of the mature Env trimer is a prerequisite for HIV-1 neutralization by antibodies (29–32). During natural HIV-1 infection, most antibodies are elicited against nonfunctional forms of Env and therefore exhibit little or no neutralizing activity (33–35). In a minority of individuals infected by HIV-1 for several years, antibodies that broadly neutralize a variety of HIV-1 strains are generated (35–42). Broadly neutralizing antibodies that recognize multiple Env conformations or that exhibit preferential recognition of state 1 or states 2/3 have been identified (14, 15).

Proteolytic processing of the gp160 Env precursor can influence antibody (Ab) binding (43–51). Many broadly neutralizing antibodies recognize the proteolytically cleaved Env trimer as well as or better than the uncleaved Env trimer, whereas weakly neutralizing antibodies efficiently recognize only the uncleaved Env trimer (43, 46–52). Changes in HIV-1 Env antigenicity associated with proteolytic processing resemble those resulting from glutaraldehyde (GA) fixation, suggesting that gp160 cleavage leads to a decrease in the conformational flexibility of the membrane Env trimer (50, 53).

Soluble HIV-1 gp140 glycoproteins that lack the transmembrane region of Env have been produced for structural characterization and vaccine studies (54–70). The effect of proteolytic processing on Env structure has been investigated in the context of these secreted Envs. Cleaved soluble gp140 (sgp140) glycoproteins require a stabilizing artificial gp120-gp41 disulfide bond, an unnatural I559P change in gp41, and a truncation of the gp41 ectodomain at residue 664 (the SOSIP.664 changes) to form compact, homogeneous trimers (60–65, 68–70). Uncleaved soluble gp140 Envs from most HIV-1 strains are secreted as monomers, dimers, trimers, and higher-order aggregates (55–60). These uncleaved soluble gp140 Envs, with or without the SOSIP changes, exhibit a heterogeneous variety of irregular shapes (68–70). Moreover, uncleaved soluble gp140 Envs are extensively modified by complex carbohydrates, in contrast to cleaved or uncleaved membrane Envs or soluble gp140 SOSIP.664 trimers, all of which primarily contain high-mannose glycans (71, 72). It has been suggested that the gp120 subunits of these uncleaved soluble gp140 glycoproteins are only loosely associated with the trimeric gp41 subunits (68, 73). In contrast, the soluble gp140 glycoproteins from selected HIV-1 strains have been reported to form more stable trimers and have been further stabilized by heterologous trimerization motifs (66, 67, 74). Based on hydrodynamic properties, the effects of insertions of flexible linkers at the gp120-gp41

FIG 1 HIV-1 Env constructs. The HIV-1_{AD8} Envs used in this study are depicted. The wild-type Env is shown, with the gp120 and gp41 transmembrane region (TM) and cytoplasmic tail (CT) indicated. The amino acid residues are numbered by alignment with the prototypic HIV-1_{HXB2} sequence, according to convention (123). The natural REKR sequence at the gp120-gp41 cleavage site is replaced in the Env(-)Δ712 glycoprotein by SEKS, eliminating proteolytic processing of the Env precursor. The sgp140 SOSIP.664 glycoproteins in the study were derived from HIV-1_{AD8} and HIV-1_{BG505}. The sgp140 SOSIP.664 Envs are modified by a disulfide bond between gp120 residue 501 and gp41 residue 605, the I559P change, substitution of an (Arg)₆ sequence at the gp120-gp41 cleavage site, and truncation after residue 664.

junction, and negative-stain electron microscopy (EM) imaging, these uncleaved soluble gp140 glycoproteins have been suggested to form compact trimers (67, 74).

Here, we investigate the effects of proteolytic cleavage on the structure of the membrane-anchored HIV-1 Env complex. We specifically address the relationship between proteolytic processing and conformational heterogeneity of the HIV-1 membrane Env. The characteristics of the membrane Envs and stabilized soluble gp140 trimers are compared.

RESULTS

Effect of cytoplasmic-tail truncation on HIV-1_{AD8} Env conformation. Truncation of the long cytoplasmic tail of gp41 results in increased levels of Env on the surfaces of expressing cells (75–78) but can decrease virus infectivity (79–83) and affect the binding of some antibodies to the Env ectodomain (16, 84–86). We evaluated the potential impact of cytoplasmic-tail truncation on the conformation of the functional Env from the AD8 strain of HIV-1 (HIV-1_{AD8}). We studied antibody neutralization of viruses with the wild-type (WT) HIV-1_{AD8} Env and HIV-1_{AD8} Env(+) Δ 712, which has a complete deletion of the gp41 cytoplasmic tail (Fig. 1). When the two virus preparations were normalized for reverse transcriptase (RT) activity, the infectivity of Env(+) Δ 712 HIV-1_{AD8} was 90% of that of the WT virus (data not shown). The WT virus and Env(+) Δ 712 HIV-1_{AD8} exhibited comparable sensitivities to neutralization by a panel of antibodies (Fig. 2). In particular, the Env(+) Δ 712 virus was as sensitive as the WT virus to antibodies like VRC01, VRC03, PG9, and PG16, which exhibit some preference for state 1 (15) (Fig. 2). Conversely, the Env(+) Δ 712 virus was as resistant as the WT HIV-1_{AD8} to antibodies like F105, 19b, 902090, 830A, and 17b, which preferentially recognize state 2 or state 3 (15) (Fig. 2 and Table 1). Thus, deletion of the gp41 cytoplasmic tail does not perceptibly alter the conformation of the functional HIV-1_{AD8} Env. These results are consistent with the minimal effects of cytoplasmic-tail deletion on the antigenicity of cell surface Envs from some primary HIV-1 strains (84, 87).

As changes in the gp41 cytoplasmic tail have been reported to influence HIV-1 Env responsiveness to ligand binding (79, 80), we tested the sensitivity of viruses with WT Env and Env(+) Δ 712 to inhibition by the small-molecule CD4-mimetic compound BNM-III-170 (88). Both viruses were efficiently inhibited by BNM-III-170 (Table 1). CD4-mimetic compounds also sensitize primary HIV-1 to neutralization by antibodies that recognize state 2 and state 3 conformations of Env (89, 90). After incubation with subneutralizing concentrations of BNM-III-170, viruses with WT Env and Env(+) Δ 712 were both efficiently inhibited by the 19b, 17b, and 902090 antibodies and by the Fab fragment of the 830A antibody (Table 1). Apparently, the WT HIV-1_{AD8} Env and HIV-1_{AD8} Env with the cytoplasmic tail deleted respond similarly to the binding of ligands, including CD4-mimetic compounds.

FIG 2 Antibody neutralization of recombinant HIV-1 with wild-type HIV-1_{AD8} Env and Env $\Delta 712$. Recombinant, luciferase-expressing HIV-1 pseudotyped with the HIV-1_{AD8} wild-type Env or Env $\Delta 712$ with the cytoplasmic tail truncated was incubated with the indicated concentrations of antibodies for 60 min at 37°C and then added to Cf2Th-CD4/CCR5 cells. Control viruses were pseudotyped with the amphotropic murine leukemia virus (A-MLV) Env. After 48 h of incubation, the cells were lysed and luciferase activity was measured. The luciferase activity in the presence of an antibody is shown relative to the luciferase activity observed in the absence of the antibody. The results of a typical set of experiments with triplicate samples are shown. The experiments were repeated with comparable results. The error bars indicate standard deviations.

TABLE 1 Sensitization of HIV-1 with wild-type Env and Env with the cytoplasmic tail deleted to antibody neutralization by a CD4-mimetic compound, BNM-III-170

Antibody ^a	gp120 epitope	IC ₅₀ (μg/ml) ^b			
		WT HIV-1 _{AD8} Env		HIV-1 _{AD8} EnvΔ712	
		+DMSO	+30 μM BNM-III-170	+DMSO	+30 μM BNM-III-170
830A Fab	V2i	>30	0.3	>30	0.3
902090	V2 linear	>30	0.5	>30	0.5
19b	V3	>30	0.2	>30	0.2
17b	CD4 induced	>30	0.2	>30	0.2

^aThe antibodies tested recognize HIV-1 gp120 epitopes that are better exposed after CD4 binding. The 830A antibody recognizes a V2 β-barrel (125). The 902090 antibody recognizes a V2 peptide spanning residues 171 to 177 (89). The 19b antibody recognizes a discontinuous V3 region epitope (126). The 17b antibody recognizes a CD4-induced discontinuous gp120 epitope overlapping the CCR5-binding site (99, 127).

^bRecombinant luciferase-expressing HIV-1 pseudotyped with either the wild-type HIV-1_{AD8} Env or HIV-1_{AD8} EnvΔ712 was incubated with DMSO or a CD4-mimetic compound, BNM-III-170 (30 μM), for 30 min at 37°C. The indicated antibodies were then added at various concentrations and further incubated at 37°C for 30 min. The mixtures were added to Cf2Th-CD4/CCR5 cells, and after a 48-hour incubation at 37°C, the cells were lysed and used to measure luciferase activity. The luciferase activity at a given antibody or Fab concentration was normalized to that seen in the absence of antibody. The 50% inhibitory concentrations (IC₅₀s) shown represent the means of the results of a typical experiment conducted with triplicate samples. The IC₅₀s for BNM-III-170 inhibition of viruses with the wild-type HIV-1_{AD8} Env or HIV-1_{AD8} Env(+)Δ712 were 4.6 and 8.2 μM, respectively.

Preparation and characterization of cleaved and uncleaved Envs. We investigated the effect of proteolytic cleavage on the HIV-1 membrane Env trimeric complex. The HIV-1_{AD8} Env(+)Δ712 glycoprotein, which has an intact proteolytic cleavage site, and Env(−)Δ712, which has an altered, nonfunctional cleavage site, were expressed transiently in human osteosarcoma (HOS) cells. The Env(+)Δ712 glycoprotein on the HOS cell surface was efficiently processed, whereas the Env(−)Δ712 glycoprotein was not (Fig. 3A and B). The HOS cells expressing the Env(+)Δ712 and Env(−)Δ712 glycoproteins were treated with glutaraldehyde to fix the Env conformation (Fig. 3C). These Envs, which were purified from solubilized HOS cell membranes, migrated as single peaks on a gel filtration column, with elution volumes consistent with those expected for trimers (~435 kDa) (Fig. 3D and E). The glutaraldehyde-fixed Env(+)Δ712 and Env(−)Δ712 glycoproteins in the trimer-associated fractions, here designated Env(+)Δ712 GA and Env(−)Δ712 GA, respectively, were characterized further in the studies described below.

Melting temperatures and hydrodynamic radii (R_h) of cleaved and uncleaved Env trimers. Using a thermal shift assay to measure the uptake of the hydrophobic dye Sypro Orange (91), the melting temperatures of the Env(+)Δ712 GA and Env(−)Δ712 GA glycoproteins were determined to be 71.5°C and 69.0°C, respectively. As expected from the stabilizing effect of glutaraldehyde fixation, these melting temperatures were higher than that of a control HIV-1_{AD8} gp120 glycoprotein that had not been cross-linked (52.5°C) (Fig. 4A).

The homogeneity of the Env(+)Δ712 GA trimer was evaluated by microscale thermophoresis. Microscale thermophoresis monitors the directed movement of a protein or protein complex in a temperature gradient, a process dependent on the size, charge, and solvation entropy of the particle (92). A thermophoretic fluorescent scan performed with the PG9 and VRC01 anti-gp120 antibodies suggested a high degree of homogeneity in the Env(+)Δ712 GA and Env(−)Δ712 GA preparations (Fig. 4B). Scans performed with soluble CD4 (sCD4) and the 2G12 antibody were also consistent with those of homogeneous Env(+)Δ712 GA and Env(−)Δ712 GA samples (data not shown).

Molecular size (the hydrodynamic radius) was calculated by coupling either Env(+)Δ712 GA or Env(−)Δ712 GA to DNA nanolevers on end-grafted electroswitchable gold nanochips (93, 94). Movement behaviors correlated with fluorescence were used to calculate dynamic responses for each protein, from which an R_h was determined (Fig. 4C). Both proteins had R_h values of approximately 10 nm.

FIG 3 Preparation of cleaved and uncleaved HIV-1_{AD8} membrane Envs. (A) HOS cells expressing the HIV-1_{AD8} Env(+) Δ 712 and Env(-) Δ 712 glycoproteins were incubated with the 2G12 anti-gp120 antibody, washed, and lysed. The lysates were incubated with protein A beads, and the precipitates were analyzed by SDS-PAGE and Western blotting. The Western blots were probed with a rabbit anti-gp120 antiserum (top) or with the 4E10 anti-gp41 antibody (124) (middle and bottom). (B) Purified membranes from HOS cells expressing the HIV-1_{AD8} Env(+) Δ 712 and Env(-) Δ 712 glycoproteins were solubilized and analyzed by Western blotting with serum from an HIV-1-infected individual. Note the efficiency with which the HIV-1_{AD8} Env(+) Δ 712 glycoprotein is cleaved. (C) Glutaraldehyde-cross-linked HIV-1_{AD8} Env(+) Δ 712 GA and Env(-) Δ 712 GA glycoproteins were purified and analyzed by SDS-PAGE and silver staining. (D) Glutaraldehyde-cross-linked HIV-1_{AD8} Env(+) Δ 712 GA and Env(-) Δ 712 GA glycoproteins were purified from HOS cell membranes and analyzed by size exclusion chromatography. The HIV-1_{AD8} gp120 Env was analyzed in parallel. The Envs were analyzed on a Yarra 4000 column equilibrated with 20 mM Tris-HCl, 150 mM NaCl, and 0.1% Cymal-6. O.D., optical density. (E) Fractions of the glutaraldehyde-cross-linked Env(+) Δ 712 GA (top) and Env(-) Δ 712 GA (bottom) glycoproteins from the Yarra 4000 size exclusion chromatography column were run on SDS-PAGE and Western blotted with serum from an HIV-1-infected individual. (Middle) The HIV-1_{AD8} Env(+) Δ 712 glycoprotein was purified from a whole-cell lysate and Western blotted as described above.

Antigenicity of the purified cleaved and uncleaved Env trimers. Proteolytic processing has been shown to affect the recognition of HIV-1 Envs by antibodies (43–52, 95–97). We examined the binding of a panel of antibodies to the purified HIV-1_{AD8} Env(+) Δ 712 GA and Env(-) Δ 712 GA glycoproteins that were captured on enzyme-linked immunosorbent assay (ELISA) plates (Fig. 5A). We also measured the affinity of antibody binding to the Env trimers in solution, using microscale thermophoresis (Fig. 5B). Soluble CD4-Ig and the neutralizing antibodies 2G12, b12, VRC01, and 4E10 bound comparably to the Env(+) Δ 712 GA and Env(-) Δ 712 GA glycoproteins. Some neutralizing antibodies, such as the quaternary-structure-dependent PG9 and PG16 antibodies (98) and the 35O22 antibody, which recognizes a gp120-gp41 epitope (95), bound more efficiently to the Env(+) Δ 712 GA glycoprotein; this is consistent with

FIG 4 Biophysical characterization of purified HIV-1_{AD8} membrane Envs. (A) Melting temperatures of the glutaraldehyde-cross-linked HIV-1_{AD8} Env(+)Δ712 GA and Env(-)Δ712 GA glycoproteins, and that of the untreated HIV-1_{AD8} gp120 control, were determined by a thermal shift assay monitoring differential Sypro Orange fluorescence. (B) Microscale thermophoresis measurements of fluorescence associated with the HIV-1_{AD8} Env(-)Δ712 GA and Env(+)Δ712 GA glycoproteins incubated with increasing concentrations (0.061 to 1,000 nM) of the PG9 (top) or VRC01 (bottom) antibody. The thermoscan patterns show no evidence of aggregation of the purified Envs. (C) R_h values of the HIV-1_{AD8} Env(+)Δ712 GA and Env(-)Δ712 GA glycoproteins immobilized on a DNA lever chip calculated from the dynamic-response values.

previous observations suggesting that these antibodies exhibit some preference for proteolytically mature HIV-1 Env trimers (50, 95). The CD4-induced (CD4i) antibodies 17b and E51, which recognize gp120 epitopes formed and exposed only after CD4 binding (99–101), bound the Env(+)Δ712 GA and Env(-)Δ712 GA trimers relatively

FIG 5 Antigenic profiles of the cleaved and uncleaved HIV-1_{AD8} Env trimers. (A) The purified Env(+) $\Delta 712$ GA and Env(-) $\Delta 712$ GA glycoproteins were incubated with saturating concentrations of the indicated antibodies in an ELISA. (Inset) The broadly neutralizing antibodies in red, mapped onto a cryoelectron tomographic map of an HIV-1 virion Env trimer (EMD-5019) (109). The envelope glycoprotein regions recognized by the antibodies (black) are depicted on the surface of an HIV-1 Env trimer. *, $P < 0.05$. The error bars indicate standard deviations. (B) Microscale thermophoresis was used to determine K_d values for the binding of the indicated antibodies to the purified Env(+) $\Delta 712$ GA and Env(-) $\Delta 712$ GA glycoproteins. At the concentrations tested, the 17b antibody did not detectably bind either glycoprotein.

inefficiently. The 19b antibody, which recognizes a gp120 V3 epitope exposed on Envs in states 2 and 3 but not in state 1 (15), bound the Env(-) $\Delta 712$ GA glycoprotein better than the Env(+) $\Delta 712$ GA glycoprotein. Likewise, the weakly neutralizing F105 antibody directed against the CD4-binding site (CD4BS) bound the Env(-) $\Delta 712$ GA glycoprotein slightly better than the Env(+) $\Delta 712$ GA glycoprotein. In contrast to the potentially neutralizing CD4BS antibodies like VRC01, F105 preferentially binds to state 2 Envs (15). Thus, relative to the uncleaved Env(-) $\Delta 712$ GA glycoprotein, the cleaved Env(+) $\Delta 712$ GA glycoprotein exhibits an antigenic profile closer to that of state 1.

Counterselection of purified membrane Env trimers with weakly neutralizing antibodies. We used weakly neutralizing antibodies that preferentially recognize state 2 or state 3 to enrich state 1 in purified preparations of the glutaraldehyde-cross-linked HIV-1 Env(+) $\Delta 712$ GA trimer (Fig. 6A). The glutaraldehyde-cross-linked Env(+) $\Delta 712$ GA glycoprotein preparation was incubated with a mixture of the 19b anti-V3 antibody and

FIG 6 Counterselection of the glutaraldehyde-fixed, purified HIV-1_{AD8} Env trimers with weakly neutralizing antibodies. (A) Workflow for negative selection of the HIV-1_{AD8} Env(+) Δ 712 GA glycoprotein by a mixture of the weakly neutralizing antibodies b6, F105, and 19b. (B) Binding of antibodies to the purified Env(+) Δ 712 GA glycoprotein and the Env(+) Δ 712 GA glycoprotein counterselected by the b12, F105, and 19b antibody mixture was evaluated in an ELISA. *, $P < 0.05$. The error bars indicate standard deviations.

the F105 and b6 weakly neutralizing CD4BS antibodies. The antigenicity of the fraction of the Env(+) Δ 712 GA glycoproteins that did not bind these antibodies was evaluated by ELISA. Counterselection resulted in a reduction in recognition of the cross-linked Env(+) Δ 712 glycoproteins by the 19b and F105 antibodies (Fig. 6B).

Effect of proteolytic cleavage on the antigenicity of cell surface Env. We also examined the effect of proteolytic cleavage on the antigenicity of the HIV-1_{AD8} Env in its membrane-anchored form on cell surfaces. The Env(+) Δ 712 glycoproteins on the surfaces of transfected HOS or HEK 293T human embryonic kidney cells were incubated with different monoclonal antibodies. After washing, the cells were lysed, and the bound antibodies were captured on protein A-Sepharose beads. The coprecipitated Envs were Western blotted. A mixture of the 2G12 and b12 anti-gp120 antibodies and CD4-Ig precipitated only gp120 and not the uncleaved Env precursor from the surfaces of Env(+) Δ 712-expressing HOS cells, consistent with the high efficiency of Env cleavage in these cells (50) (Fig. 7A, top). The gp120 glycoprotein of the cell surface Env(+) Δ 712 trimers was precipitated by the broadly neutralizing antibodies VRC01, PG9, and 35O22, but not by the weakly neutralizing 19b anti-V3 antibody or the CD4BS antibody F105. Thus, broadly neutralizing antibodies recognize the mature Env trimer and precipitate gp120, whereas weakly neutralizing antibodies that exhibit a preference for state 2 do not.

We also examined antibody recognition of cell surface Env(+) Δ 712 trimers transiently expressed in HEK 293T cells, where Env cleavage is less efficient than in HOS cells (50) (duplicate experiments are shown in Fig. 7A, bottom). In this case, both cleaved and uncleaved Env(+) Δ 712 glycoproteins are available for antibody binding and precipitation. The potent CD4BS-neutralizing antibody VRC01 precipitated both

FIG 7 Antigenic profile of the HIV-1_{AD8} Env(+) Δ 712 glycoprotein displayed on the surfaces of expressing cells. (A) The HIV-1_{AD8} Env(+) Δ 712 glycoprotein was expressed in HOS cells, where gp120-gp41 cleavage is efficient, and in HEK 293T cells, where proteolytic cleavage is incomplete. The indicated antibodies were incubated with the Env(+) Δ 712-expressing cells, which were washed and then lysed. The cell lysates were incubated with protein A beads, and the precipitates were Western blotted (WB) with serum from an HIV-1-infected individual. The antibodies used for the cell surface immunoprecipitation included broadly neutralizing antibodies (VRC01, PG9, and 35O22) and weakly neutralizing antibodies (19b and F105). The mixture of 2G12, CD4-Ig, and b12 was used to precipitate all forms of Env(+) Δ 712 from the cell surface. (B) A cell-based ELISA with the indicated antibodies was used to evaluate the effect of glutaraldehyde treatment on the antigenicity of the HIV-1_{AD8} Env(+) Δ 712 glycoprotein expressed on the surfaces of HOS cells. The error bars indicate standard deviations.

cleaved and uncleaved Envs. The PG9 and 35O22 antibodies preferentially precipitated the cleaved Env(+) Δ 712 glycoproteins. In contrast, the 19b and F105 antibodies precipitated only the uncleaved Env(+) Δ 712 glycoproteins. Our results suggest that the cleaved HIV-1_{AD8} membrane Env mainly populates state 1 and is not recognized by weakly neutralizing antibodies with a preference for state 2 or state 3. The uncleaved Env samples conformations other than state 1 more often than the cleaved Env.

Effect of glutaraldehyde cross-linking on the antigenicity of cell surface Env trimers. We investigated the effect of glutaraldehyde treatment on the antigenicity of the HIV-1_{AD8} Env(+) Δ 712 trimers expressed on HOS cell surfaces, using a cell-based ELISA (17). Although the binding of most of the tested antibodies was increased after

glutaraldehyde cross-linking, the general pattern of Env recognition by the different antibodies was similar to that of the untreated Env(+) Δ 712-expressing cells (Fig. 7B). Glutaraldehyde treatment was associated with increases in the binding of several potentially neutralizing antibodies (VRC01, PG9, PG16, PGT128, PGT121, 35O22, and 4E10), as well as some weakly neutralizing antibodies (the 19b anti-V3 antibody and the 902090 anti-V2 antibody). Another V3-directed antibody, 39F, bound inefficiently to both the untreated and cross-linked HIV-1_{AD8} Env(+) Δ 712 glycoproteins. These data suggest that glutaraldehyde treatment preserves the overall antigenicity of the HIV-1_{AD8} Env(+) Δ 712 glycoprotein but may result in local conformational changes in the V3 and V2 regions of gp120 and the gp41 membrane-proximal external region. These results are consistent with those obtained in other studies of cross-linked HIV-1 Envs (17, 50, 53, 102).

Characterization of soluble gp140 SOSIP.664 Env trimers produced in HOS cells. Stabilized soluble versions of the cleaved HIV-1 Env trimer were created by deletion of the gp41 transmembrane region and cytoplasmic tail, as well as a portion of the gp41 ectodomain near the viral membrane; these soluble trimers were stabilized by the I559P modification of the gp41 ectodomain and the introduction of an artificial disulfide bond linking gp120 and gp41 (60–65, 68). We produced sgp140 SOSIP.664 glycoproteins from HIV-1_{BG505} (Fig. 8) and HIV-1_{AD8} (Fig. 9) in HOS cells. The secreted sgp140 SOSIP.664 glycoproteins underwent very efficient cleavage (Fig. 8A and 9A), migrated homogeneously on a size exclusion column at a size expected for a trimer (Fig. 8B and 9B), and exhibited characteristic compact trimer structures by negative-stain electron microscopy (Fig. 8C and 9C). Microscale thermophoresis fluorescent scans (92) with the PG9 anti-gp120 antibody suggested a high degree of homogeneity in the HIV-1_{BG505} and HIV-1_{AD8} sgp140 SOSIP.664 samples (Fig. 8D and 9D).

The molecular size (R_h) of the sgp140 SOSIP.664 glycoproteins was calculated by conjugating the Env trimers to DNA nanolevers and monitoring dynamic responses for each Env protein (93, 94) (Fig. 8E and 9E). The R_h of 8.05 nm calculated for the HIV-1_{BG505} sgp140 SOSIP.664 trimer is consistent with the experimental R_h value of 8.1 nm obtained previously by small-angle X-ray scattering (103). Consistent with these results, the R_h of the HIV-1_{AD8} sgp140 SOSIP.664 trimers was calculated to be 7.93 nm (Fig. 9E).

Determination of the binding constants by microscale thermophoresis showed high affinities of 2G12, VRC01, sCD4, and PG9 for the HIV-1_{BG505} and HIV-1_{AD8} sgp140 SOSIP.664 trimers (Fig. 8F and 9F and data not shown). Three-dimensional reconstructions derived from negative-stain electron microscopy images revealed compact trimers similar to those published previously (62, 64, 65, 68) (Fig. 8G and H and 9G and H).

Antigenicity of untreated and glutaraldehyde-cross-linked soluble gp140 SOSIP.664 Env trimers and cell surface Envs. We evaluated the antigenicity of the purified untreated and glutaraldehyde-cross-linked HIV-1_{BG505} and HIV-1_{AD8} soluble gp140 SOSIP.664 glycoproteins. The antigenic profile of the sgp140 SOSIP.664 Envs was compared with that of the membrane Envs on cell surfaces (Fig. 10 and 11). Both sgp140 SOSIP.664 trimers, either untreated or glutaraldehyde treated, were recognized efficiently in an ELISA by several antibodies (2G12, VRC01, 3BNC117, PGT121, and PGT128) that neutralized viruses containing the corresponding wild-type Envs (Fig. 2, Table 2, and data not shown). Among the antibodies recognizing the gp120 CD4BS, the most potent neutralizing antibodies, 3BNC117 and VRC01, bound the sgp140 SOSIP.664 Envs most efficiently. However, for the CD4BS antibodies that neutralized HIV-1_{BG505} less potently, no correlation with binding to the HIV-1_{BG505} sgp140 SOSIP.664 glycoprotein was evident. For example, the b12 and b6 antibodies bound sgp140 SOSIP.664 better than VRC03, VRC13, and VRC16, all three of which neutralized HIV-1_{BG505} more potently (Table 2). The sgp140 SOSIP.664 glycoproteins were also recognized by antibodies (19b and 1.4e) against the gp120 V3 region that did not neutralize the cognate viruses. Likewise, the 902090 anti-V2 antibody bound the HIV-1_{BG505} sgp140 SOSIP.664 Env but did not efficiently neutralize viruses with the HIV-1_{BG505} Env (Fig. 10A and E and Table 2). We note that the nonneutralizing antibodies (b6, F105, 17b, 830A,

FIG 8 Purification and characterization of the HIV-1_{BG505} soluble gp140 SOSIP.664 Env trimer. (A) SDS-PAGE and Coomassie blue staining of the HIV-1_{BG505} sgp140 SOSIP.664 Env produced in HOS cells. The sgp140 SOSIP.664 Env preparations were treated with dithiothreitol (DTT) or glutaraldehyde (GA) or left untreated prior to analysis on the gel. (B) Purified sgp140 SOSIP.664 Env was analyzed by size exclusion chromatography. (C) Reference-free two-dimensional (2D) class averages of negative-stained sgp140 SOSIP.664 trimers. (D) Microscale thermophoresis analysis of the binding of the PG9 antibody was used to assess the aggregation of the sgp140 SOSIP.664 trimers. (E) R_h values of the sgp140 SOSIP.664 trimers determined by immobilization on a DNA lever chip and calculated from dynamic-response values. (F) K_d values of the indicated antibodies/ligands with the glutaraldehyde-cross-linked sgp140 SOSIP.664 trimer determined by microscale thermophoresis. (G) Three-dimensional model of the HIV-1_{BG505} sgp140 SOSIP.664 trimer derived from the 2D class averages shown in panel C. (H) Resolution of the negative-stain EM reconstruction shown in panel G estimated by Fourier shell correlation, using a cutoff of 0.5.

19b, 1.4e, and 902090) did not efficiently bind the HIV-1_{AD8} Env(+) Δ 712 glycoproteins on the surfaces of transfected cells (Fig. 10A and 11A). As some nonneutralizing antibodies do not recognize state 1 but do bind sgp140 SOSIP.664 Envs, at least a fraction of the sgp140 SOSIP.664 trimers must sample conformations distinct from state

FIG 9 Purification and characterization of the HIV-1_{AD8} soluble gp140 SOSIP.664 Env trimer. (A) The HIV-1_{AD8} sgp140 SOSIP.664 Env produced in HOS cells was analyzed by SDS-PAGE and Western blotting with serum from an HIV-1-infected individual. The sgp140 SOSIP.664 Env preparations were untreated or treated with GA prior to analysis on the gel. (B) The purified sgp140 SOSIP.664 Env was analyzed by size exclusion chromatography. (C) Negative-stain electron micrograph of purified HIV-1_{AD8} sgp140 SOSIP.664 Env trimers. Scale bar, 50 nm. (D) Microscale thermophoresis analysis of the binding of the PG9 antibody was used to assess the aggregation of the sgp140 SOSIP.664 trimers. (E) R_h values of the sgp140 SOSIP.664 trimers determined by immobilization on a DNA lever chip and calculated from dynamic-response values. (F) K_d values of the indicated antibodies/ligands with the glutaraldehyde-cross-linked sgp140 SOSIP.664 trimer determined by microscale thermophoresis. (G) Three-dimensional model of the HIV-1_{BG505} sgp140 SOSIP.664 trimer derived by reconstruction from negative-stain images. (H) Resolution of the negative-stain EM reconstruction shown in panel G estimated by Fourier shell correlation using a cutoff of 0.5.

FIG 10 Comparison of the antigenic profiles of the HIV-1_{BG505} sgp140 SOSIP.664 Env trimers and HIV-1_{AD8} Envs on cell surfaces. (A to D) Binding of the indicated antibodies to the HIV-1_{BG505} sgp140 SOSIP.664 trimers, relative to that of the 2G12 antibody, captured on an ELISA plate. The sgp140 SOSIP.664 trimers were either untreated (A and C) or cross-linked with glutaraldehyde (B and D) prior to the ELISA. For comparison, the binding of the same set of antibodies to the HIV-1_{AD8} Env(+) Δ 712 or Env(-) Δ 712 glycoproteins, either untreated or glutaraldehyde treated, on the surfaces of HOS cells was evaluated by cell-based ELISA. The percentages of the binding observed for the 2G12 antibody are shown. *, $P < 0.05$. (E) SDS-PAGE of HIV-1_{BG505} sgp140 SOSIP.664 trimers precipitated by the indicated antibodies and Western blotted with an anti-gp120 HRP rabbit polyclonal antibody. Note that the inefficient recognition of the sgp140 SOSIP.664 Env by the PGT151 antibody is specific to the ELISA format, as PGT151 could recognize sgp140 SOSIP.664 trimers in the immunoprecipitation assay. The error bars indicate standard deviations.

1. The epitopes for several of these poorly neutralizing antibodies are located in the gp120 trimer association domain, which is composed of the V1/V2 and V3 variable regions (84, 103–106). Apparently, there are differences in the conformations of the V1/V2 and V3 regions of the soluble gp140 SOSIP.664 glycoprotein and native HIV-1 Env

FIG 11 Comparison of the antigenic profiles of the HIV-1_{AD8} sgp140 SOSIP.664 Env trimers and HIV-1_{AD8} Env(+)/Δ712 on cell surfaces. (A and B) Binding of the indicated antibodies to the HIV-1_{AD8} sgp140 SOSIP.664 trimers captured on an ELISA plate, relative to that of the 2G12 antibody. The sgp140 SOSIP.664 trimers were either untreated (A) or cross-linked with glutaraldehyde (B) prior to the ELISA. For comparison, the binding of the same set of antibodies to the HIV-1_{AD8} Env(+)/Δ712 glycoprotein, either untreated or glutaraldehyde treated, on the surfaces of HOS cells was evaluated by cell-based ELISA. The percentages of the binding observed for the 2G12 antibody are shown. *, $P < 0.05$. The error bars indicate standard deviations.

trimers in state 1. The quaternary structure of the HIV-1 Env trimer has been shown to influence the access of CD4BS antibodies to their gp120 epitopes (46, 52, 84, 107). The poor correlation between sgp140 SOSIP.664 binding and neutralization of some CD4BS antibodies may reflect differences in the quaternary orientations of the gp120 protomers in the soluble trimers and virion spikes.

Glutaraldehyde cross-linking has been used to attempt to further stabilize the soluble gp140 SOSIP.664 trimers for use as immunogens (53, 102). We treated the HIV-1_{BG505} and HIV-1_{AD8} sgp140 SOSIP.664 glycoproteins with glutaraldehyde and tested the binding of antibodies in an ELISA. The binding of several nonneutralizing antibodies (b6, F105, 17b, 19b, and 1.4e) was decreased by glutaraldehyde treatment (Fig. 10B and 11B). Several neutralizing antibodies (VRC01, 3BNC117, PG9, PGT16,

TABLE 2 Antibody neutralization of recombinant HIV-1 with wild-type HIV-1_{BG505} Env

Antibody	Env epitope	IC ₅₀ (μg/ml) ^a
3BNC117	CD4-binding site	0.15
VRC01	CD4-binding site	0.46
VRC03	CD4-binding site	1.67
VRC16	CD4-binding site	1.80
VRC13	CD4-binding site	3.31
b12	CD4-binding site	>10
b6	CD4-binding site	>10
F105	CD4-binding site	>10
PG9	V2 quaternary	0.31
PG16	V2 quaternary	0.12
PGT145	V2 quaternary	0.16
830A	V2i	>10
902090	V2 linear	~10
19b	V3	>10
PGT121	V3 glycan	0.089
PGT128	V3 glycan	<0.01
2G12	Outer-domain glycan	1.20
17b	CD4i	>10
PGT151	gp120-gp41	0.002

^aRecombinant luciferase-expressing HIV-1 pseudotyped with the wild-type HIV-1_{BG505} Env was incubated with the indicated antibody for 1 h at 37°C. The virus-antibody mixture was spinoculated on Cf2Th-CD4/CCR5 cells at 800 × *g* for 1 h at room temperature. After a 48-hour incubation at 37°C, the cells were lysed, and luciferase activity was measured. The reported IC₅₀s represent the means of the results of at least two experiments conducted with triplicate samples.

FIG 12 Negative-stain electron microscopy of cleaved and uncleaved HIV-1_{AD8} membrane Envs. Micrographs of negative-stained HIV-1_{AD8} Env(+) Δ 712 GA (A) and Env(-) Δ 712 GA (B) glycoprotein trimers are shown. (C and D) Reference-free class averages of the Env(+) Δ 712 GA (C) and Env(-) Δ 712 GA (D) trimers.

PGT121, PGT128, and 2G12) bound the glutaraldehyde-cross-linked sgp140 SOSIP.664 trimers. Thus, cross-linking the soluble gp140 SOSIP.664 trimers with glutaraldehyde resulted in an antibody-binding profile that correlated better with the virus neutralization profile.

Negative-stain EM of cleaved HIV-1 Env trimers. The antigenic profile of the purified HIV-1_{AD8} Env(+) Δ 712 glycoprotein resembles that expected for an Env trimer populating state 1, and counterselection with weakly neutralizing antibodies can further reduce the fraction of Env(+) Δ 712 trimers in the preparation that sample conformations other than state 1. To gain insights into the conformations of the functional HIV-1_{AD8} Env trimer, we collected negative-stain electron microscopy images of the glutaraldehyde-fixed Env(+) Δ 712 glycoproteins (Fig. 12A and C). Negative-stain electron microscopy images of the uncleaved HIV-1_{AD8} Env(-) Δ 712 GA glycoproteins prepared in parallel showed greater heterogeneity (Fig. 12B and D); consistent with this apparent heterogeneity, attempts to reconstruct a model from the Env(-) Δ 712 GA class averages failed.

A negative-stain EM reconstruction of the glutaraldehyde-cross-linked HIV-1_{AD8} Env(+) Δ 712 trimer, after counterselection with the 19b, b6, and F105 antibodies, is shown in Fig. 13A to E. At a resolution of approximately 22 Å, the Env(+) Δ 712 trimer exhibits a hollow architecture with a central void near the trimer axis (Fig. 13D). This architecture is similar to that previously reported for membrane-anchored Envs in HIV-1 virions (by cryoelectron tomography) and on cell surfaces (by single-particle cryoelectron microscopy) (84, 108–111). In contrast, the negative-stain reconstructions of the sgp140 SOSIP.664 glycoproteins showed more compact trimers with dense cores along the trimer axis (Fig. 8G, 9G, and 13F). Our sgp140 SOSIP.664 density maps are consistent with the published negative-stain EM maps, cryo-EM density maps, and X-ray crystal structures of sgp140 SOSIP.664 trimers (64, 65, 68–70, 73, 103–105, 112). These results

FIG 13 Negative-stain electron microscopy reconstruction of the HIV-1_{AD8} Env(+) Δ 712 GA trimer after counterselection. (A) Micrograph of negative-stained HIV-1_{AD8} Env(+) Δ 712 GA derived by counterselection with the 19b, b6, and F105 antibodies. Scale bar, 50 nm. (B) Reference-free class averages derived from micrographs of negative-stained HIV-1_{AD8} Env(+) Δ 712 GA glycoproteins purified from cell membranes. (C) The resolution of the reconstruction shown in panel B was estimated by Fourier shell correlation, with a cutoff of 0.5. (D) Three-dimensional structural model of the HIV-1_{AD8} Env(+) Δ 712 GA trimer derived from the class averages in panel B. (E) Comparisons of 3D model-derived projections, class averages, and single-particle images of the Env(+) Δ 712 trimer. (F) The HIV-1_{AD8} Env(+) Δ 712 GA trimer (gray) reconstruction was aligned with the sgp140 SOSIP.664 trimers from HIV-1_{BG505} (purple) and HIV-1_{AD8} (yellow).

suggest that differences exist between the conformations of cleaved unliganded HIV-1 Env trimers anchored in viral and cell membranes and sgp140 SOSIP.664 trimers (Fig. 13F).

DISCUSSION

The metastable, functional Env trimers from primary HIV-1 strains reside in a local energy well in a conformation (state 1) that is separated by a relatively high activation barrier from an obligate intermediate conformation (state 2) and the CD4-bound conformation (state 3) (14–16, 113). CD4 binding or alteration of particular restraining residues in Env that are involved in maintaining state 1 lowers the activation barrier and releases Env to sample downstream conformations. As state 1 represents a major target for inhibitors, including antibodies, understanding this Env conformation is a high priority. Investigation of state 1 requires approaches that avoid premature triggering. In this study, we evaluated the impact on the Env conformation of several modifications that are often used to facilitate production, purification, and characterization of HIV-1 Env trimers.

Truncation of the approximately 150-residue gp41 cytoplasmic tail has been reported to affect the antigenicity of the Env ectodomain (85, 86) and may decrease the occupancy of potential N-linked glycosylation sites on Env (71). We found that complete deletion of the gp41 cytoplasmic tail had little to no impact on the sensitivity of HIV-1_{AD8} to a CD4-mimetic compound or to neutralization by multiple antibodies. Thus, at least for the functional HIV-1_{AD8} Env, the cytoplasmic tail is not required for maintenance of a state 1 conformation.

Expression of the HIV-1_{AD8} Env in cells in which the efficiency of gp120-gp41 cleavage varied allowed an assessment of the impact of proteolytic maturation on Env conformation. In HOS cells, the surface Env(+) Δ 712 glycoproteins are efficiently cleaved and resist the binding of weakly neutralizing antibodies (19b and F105) that recognize Env conformations other than state 1 (e.g., state 2 and state 3) (15, 113). In HEK 293T cells, where Env cleavage is inefficient, these weakly neutralizing antibodies bound only the uncleaved Env. These results indicate that the majority of the mature HIV-1_{AD8} Env resides in conformational state 1, a conclusion that is consistent with single-molecule fluorescence resonance energy transfer (smFRET) studies of other primary HIV-1 Envs (14, 15, 113). In contrast, the uncleaved HIV-1_{AD8} Env trimer on the cell surface samples conformations, including states 2 and 3, not sampled by the cleaved Env.

We directly compared the antigenicity of the HIV-1_{AD8} cleaved Env(+) Δ 712 and uncleaved Env(-) Δ 712 glycoproteins, fixing the Envs with glutaraldehyde in an attempt to preserve their unliganded conformations throughout purification. The overall antigenic profiles of the purified Env(+) Δ 712 and Env(-) Δ 712 glycoproteins are similar, indicating that the organization of the gp120 and gp41 subunits in the uncleaved Env trimer approximates that in the cleaved Env trimer. This result is consistent with the observation that the single-particle cryo-EM structure of the uncleaved HIV-1_{JR-FL} Env(-) Δ 712 trimer purified from expressing cell membranes is indistinguishable at ~25- to 30-Å resolution from the tomograms of the proteolytically mature HIV-1 virion Env spike (84, 108–111). Our result is also consistent with the similarity of the effects of glutaraldehyde fixation on the antigenicity of cleaved and uncleaved forms of cell surface Env (50). Given that proteolytic maturation is a very late event in the biosynthesis of HIV-1 Env trimers (20–27), the organizational similarity of uncleaved and mature Env complexes is intuitively satisfying, because at least some of the noncovalent interactions between the gp120 and gp41 subunits that maintain trimer integrity can be established prior to cleavage.

Conformational differences observed between the cleaved and uncleaved HIV-1_{AD8} Env trimers on the cell surface were retained in the cross-linked, purified Env(+) Δ 712 GA and Env(-) Δ 712 GA trimers. The Env(+) Δ 712 GA glycoprotein bound the PG9, PG16, and 35O22 neutralizing antibodies more efficiently than the Env(-) Δ 712 GA glycoprotein. The weakly neutralizing antibodies F105 and 19b recognized the Env(-) Δ 712 GA glycoprotein more efficiently than the Env(+) Δ 712 GA glycoprotein. Neither glycoprotein efficiently bound the CD4-induced 17b and E51 antibodies. The differences observed between the antibody recognition of the HIV-1_{AD8} Env(+) Δ 712 GA and Env(-) Δ 712 GA glycoproteins are consistent with a model in which the cleaved HIV-1 Env is less flexible than the uncleaved Env. Greater flexibility of Env(-) Δ 712 GA might explain the results of our negative-stain electron microscopy studies, where the Env(+) Δ 712 GA data set yielded a structure but the Env(-) Δ 712 GA data set failed to converge. This model also provides a natural explanation for the observation that proteolytic cleavage and glutaraldehyde fixation exerted similar effects on the recognition of cell surface HIV-1 Env trimers by a panel of antibodies (50). Like glutaraldehyde cross-linking, gp120-gp41 cleavage apparently results in a tighter interaction among the subunits of the Env trimeric complex, decreasing the propensity of Env to undergo conformational change (16, 17). As a consequence, the proteolytically mature, predominantly state 1 HIV-1 Env trimer is better able to resist the binding of nonneutralizing or weakly neutralizing antibodies, which require changes in Env conformation to bind efficiently (17, 114).

For soluble HIV-1 Env trimers, maintaining the metastable structure of the gp41 ectodomain and preserving its native interaction with gp120 in the absence of the membrane-spanning domain represent significant challenges. For the soluble gp140 Envs derived from many HIV-1 strains, lack of proteolytic processing is associated with extreme conformational heterogeneity (68, 73). Lack of cleavage in these soluble Envs, including the HIV-1_{BG505} soluble gp140 SOSIP.664 glycoprotein, has been suggested to result in trimers in which the normal association between gp120 and the gp41 ectodomain is attenuated (68). Presumably, in the context of the already weak gp120-gp41 association in the soluble gp140s, the absence of processing engenders conformational instability to a more exaggerated degree than that seen in membrane-anchored Envs.

Many antibodies with no or weak neutralizing activity recognize HIV-1 Env conformations other than state 1. Counterselection of Env preparations with these antibodies can enrich conformations like state 1 that are not efficiently bound by the antibodies; in this process, Env molecules with elements exposed as a result of spontaneous sampling of downstream conformations or fixation-related changes can be removed from the preparation. We prepared glutaraldehyde-fixed, mature HIV-1_{AD8} Env(+) Δ 712 glycoproteins from cell membranes and counterselected one-half of the preparation with a mixture of three weakly neutralizing antibodies (19b plus b6 plus F105). Counterselected samples were imaged by negative-stain EM. The three HIV-1_{AD8} Env(+) Δ 712 protomers form a cage-like structure surrounding a central void at the trimer axis. The only available structural information on the unliganded cleaved HIV-1 membrane Env derives from cryoelectron tomograms of virion surfaces (109, 110). Despite differences in EM approaches, Env hydration states, sample preparation variables, presence of the Env cytoplasmic tail, and HIV-1 strains of origin, the Env(+) Δ 712 and virion maps are consistent. The hollow, cage-like architecture of membrane Env trimers has also been observed for simian immunodeficiency virus Envs on virions (by cryoelectron tomography) and for uncleaved HIV-1_{JR-FL} Env(–) Δ 712 Envs purified from cell membranes (by single-particle cryo-EM) (84, 108, 110, 111). This hollow architecture may generally reflect the metastability of immunodeficiency virus Env trimers, which proceed to more stable downstream states (e.g., the pre-hairpin intermediate [state 3] or the six-helix bundle) by collapsing toward the trimer axis.

The global architecture of the HIV-1_{BG505} and HIV-1_{AD8} soluble gp140 SOSIP.664 trimers differs from that of the membrane Env trimers. Compared with the membrane Envs, the sgp140 SOSIP.664 trimers are more compact, as reflected in their smaller hydrodynamic radii and negative-stain EM structures. In the sgp140 SOSIP.664 Envs, the three protomers are displaced toward the trimer axis, filling the central void seen in the membrane Envs. If our hypothesized association of the hollow immunodeficiency virus Env architecture with metastability is valid, the stabilizing changes introduced into the sgp140 SOSIP.664 glycoprotein, such as the helix-breaking I559P alteration, may have resulted in the collapse of Env elements toward the trimer axis (87, 115, 116). This collapse may have exerted secondary effects on the gp120 conformation. Discrepancies between the recognition of the sgp140 SOSIP.664 trimers by several antibodies recognizing gp120 V2, V3, and CD4BS epitopes and the neutralization sensitivity of the corresponding viruses were observed. Thus, the sgp140 SOSIP.664 glycoproteins and the functional state 1 HIV-1 Env may differ in the trimer association domain and in the quaternary conformation of the trimer, which restricts access of the CD4BS antibodies to their epitopes (46, 52, 84, 107). Understanding the effects of the trimer-stabilizing changes introduced into the sgp140 SOSIP.664 glycoprotein on Env conformation could help to restore more native features to next-generation Env vaccine constructs. Such changes may be necessary for the elicitation of broadly neutralizing antibodies, which has not been achieved with current immunogens (33, 117, 118).

MATERIALS AND METHODS

Cell lines. HEK 293T and HOS cells (ATCC) were grown at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum (Sigma) and 100 μ g/ml penicillin-

streptomycin (Mediatech, Inc.). Cf2Th cells stably expressing human CCR5 and CD4 were grown in medium supplemented with 0.4 mg/ml G418 and 0.2 mg/ml hygromycin (Invitrogen).

Recombinant luciferase-expressing viruses. For the experiments shown in Fig. 2 and Table 1, 293T human embryonic kidney cells were cotransfected with plasmids expressing the pCMVΔP1Δenv HIV-1 Gag-Pol packaging construct, the HIV-1 envelope glycoproteins, and a firefly luciferase-expressing vector at a DNA ratio of 1:1:3 μ g using the Effectene transfection reagent (Qiagen). For the experiments shown in Table 2, 293T cells were transfected by the calcium phosphate method with the HIV-1 proviral vector pNL4.3Env⁺-Luc and the pSVIIenv Env-expressing plasmid at a 4:1 (wt/wt) DNA ratio. Cotransfection produced recombinant, luciferase-expressing viruses capable of a single round of infection. The virus-containing cell supernatants were harvested 36 to 40 h after transfection and cleared of debris by low-speed centrifugation. Aliquots of the virus preparations were frozen at -80°C until further use. The RT levels of all virus stocks were measured (119).

Single-round infection and neutralization assays. Cf2Th-CD4/CCR5 target cells were seeded at a density of 6×10^3 cells/well in 96-well luminometer-compatible tissue culture plates (PerkinElmer) 24 h before infection. On the day of infection, antibodies (over a range of 0 to 100 $\mu\text{g/ml}$) were mixed with the virus and incubated at 37°C for 60 min. For experiments evaluating HIV-1 sensitization by a CD4-mimetic compound, the CD4-mimetic compound BNM-III-170 (30 μM) (88) or an equivalent concentration of the dimethyl sulfoxide (DMSO) vehicle was mixed with the virus and incubated for 30 min at 37°C . Antibodies were then added, and the 37°C incubation continued for an additional 30 min. The virus-antibody or virus-compound-antibody mixtures were then added to the target cells and incubated for 48 h at 37°C ; after this time, the medium was removed from each well, and the cells were lysed by the addition of 30 μl passive lysis buffer (Promega) and three freeze-thaw cycles. An EG&G Berthold microplate luminometer LB 96V was used to measure the luciferase activity after the addition of 100 μl of luciferin buffer (15 mM MgSO_4 , 15 mM KPO_4 , pH 7.8, 1 mM ATP, and 1 mM dithiothreitol) and 50 μl of 1 mM firefly D-luciferin-free acid 99% (Prolume) to each well.

HIV-1 envelope glycoproteins. Cleavage-competent Env(+) Δ 712 and cleavage-defective Env(−) Δ 712 glycoproteins from the primary HIV-1_{AD8} strain (GenBank accession number [AF004394](#)) were transiently expressed in HOS cells, a cell line in which Env proteolytic processing is efficient (17, 50). Both Env glycoproteins have a truncated cytoplasmic tail to increase cell surface expression levels (75–78). Processing of the Env(−) Δ 712 glycoprotein was prevented by introduction of the R508S and R511S changes at the gp120-gp41 cleavage site (21, 120) (Fig. 1).

The HIV-1_{BG505} soluble gp140 6R.SOSIP.664.T332N Env trimers with a C-terminal D7324 epitope tag (here called soluble gp140 [sgp140] SOSIP.664 glycoproteins) were expressed by Effectene transfection of HOS cells with a plasmid provided by P. D. Kwong (Vaccine Research Center) (103, 121). The HIV-1_{AD8} sgp140 SOSIP.664 construct was designed by introducing cysteines at positions 501 and 605, by modifying the gp120-gp41 cleavage site to (Arg)₆, by changing isoleucine to proline at residue 559 (I559P), and by truncating gp41 at residue 664 without the addition of an epitope tag. The HIV-1_{AD8} sgp140 SOSIP.664 construct was also expressed by Effectene transfection of HOS cells.

Fixation and purification of Envs. HOS cells expressing the HIV-1_{AD8} Env(+) Δ 712 and Env(−) Δ 712 glycoproteins were treated with 5 mM glutaraldehyde for 20 min at room temperature to fix the Env conformation (17, 50, 53). Glutaraldehyde activity was halted using 100 mM Tris. Env glycoproteins were solubilized from cell membranes as previously described (108) and purified by *Galanthus nivalis* lectin affinity chromatography. Trimers of the lectin-purified Envs were enriched by size exclusion chromatography, using a Yarra 4000 column (Phenomenex) equilibrated with 20 mM Tris-HCl, 150 mM NaCl, and 0.1% Cymal-6 (Anatrace). The glutaraldehyde-fixed purified Envs were stored in this buffer at -80°C .

HIV-1_{AD8} and HIV-1_{BG505} sgp140 SOSIP.664 trimers were secreted in the supernatant of HOS cells and then purified by *Galanthus nivalis* lectin affinity chromatography and size exclusion chromatography, using a Yarra 4000 column equilibrated with 20 mM Tris-HCl and 150 mM NaCl. The sgp140 SOSIP.664 glycoproteins were concentrated and stored at -80°C .

Thermal shift assay. The melting temperatures of the HIV-1_{AD8} Env(+) Δ 712, Env(−) Δ 712, and gp120 glycoproteins were estimated by a thermal shift assay measuring differential Sypro Orange fluorescence (Invitrogen, Carlsbad, CA) (91). The Env(+) Δ 712 and Env(−) Δ 712 glycoproteins were glutaraldehyde cross-linked, whereas the control gp120 glycoprotein was not cross-linked. A real-time PCR machine (iCycler; Bio-Rad, Hercules, CA) was used to monitor protein unfolding by the increase in the fluorescence of the fluorophore Sypro Orange. Protein samples (40 μl at 3 μM) were analyzed in phosphate-buffered saline (PBS) (pH 7.5), 0.1% Cymal-6 detergent.

ELISA of purified proteins. Purified Envs were diluted in 20 mM HEPES, pH 7.4, 300 mM NaCl, and 0.1% Cymal-6 to an appropriate volume and then dispensed into the wells of a white nickel-coated 96-well plate (Pierce) and incubated overnight. The plates were washed three times with washing buffer (20 mM Tris HCl, pH 7.4, 150 mM NaCl, 0.1% Cymal-6) and then blocked with blocking buffer (10% bovine serum albumin [BSA] [Sigma] in washing buffer) for 0.5 to 2 h. The wells were washed three times with washing buffer and then incubated for 1 h with antibody diluted to 10 $\mu\text{g/ml}$ in blocking buffer. Antibodies 98-6, F240, 5F3, 240-D, 246-D, 39F, E51, PGT121, PGT128, F105, 4E10, 35O22, b6, b12, VRC01, VRC03, 412D, and 1.4E were obtained from the NIH AIDS Reagent Program. 3BNC117 was provided by M. Nussenzweig (Rockefeller University). 2G12 was purchased from Polymun. 1D4 was purchased from Abcam. Antibodies 17b, 19b, PG9, PG16, 35O22, C11, PGT151, 902090, 3BNC117, VRC13, and VRC16 were produced from transfected HEK 293F cells, with purification of the cell supernatants by protein A. Antibody concentrations were measured with a BCA kit (Thermo Fisher), and antibodies were aliquoted and stored at -80°C . For ELISA, after antibody binding, wells were washed three times with washing buffer and then incubated with protein A-horseradish peroxidase (HRP) at 1:4,000 in blocking buffer for

1 h. The wells were washed three times with washing buffer and Tris-buffered saline (TBS). The wells were then probed with West Luminol reagent Lightning ECL (PerkinElmer), and luminescence was measured with a Mithras LB 940 luminometer (Berthold Technologies).

Cell-based ELISA. HOS cells were seeded in white 96-well plates. At 50% confluence, the cells were transfected with plasmids expressing the HIV-1_{AD8} Env(+) Δ 712 and Env(-) Δ 712 glycoproteins, as previously reported (50). After 48 h of incubation, the medium was removed and the cells were washed twice in HEPES washing buffer (20 mM HEPES, 1.8 mM CaCl₂, 1 mM MgCl₂, 140 mM NaCl). The buffer was removed, and the cells were incubated with 5 mM glutaraldehyde in HEPES washing buffer for 25 min. Untreated cells were left in HEPES washing buffer. The cells were washed with 50 mM Tris HCl, pH 7.4, 1.8 mM CaCl₂, 1 mM MgCl₂, and 140 mM NaCl to quench the cross-linking reaction. A cell-based ELISA was then performed as described previously (50). The cells were probed with HRP enzyme oxidizing and Luminol reagents (PerkinElmer Life Sciences) supplemented with 150 mM NaCl. Luminescence was measured with a Mithras LB 940 luminometer (Berthold Technologies). The results of the cell-based ELISAs from six independent experiments were averaged. Luminescence values for the different antibodies used were then normalized to the signal obtained with the 2G12 antibody.

Negative selection of purified Env. The glutaraldehyde-cross-linked HIV-1_{AD8} Env(+) Δ 712 glycoprotein was further purified by negative selection. The protein was incubated with a 10-fold molar excess of a b6, F105, and 19b antibody mixture and incubated for 2 h at room temperature at a final concentration of 0.05 mg/ml. Reaction mixtures were diluted 50-fold in 20 mM HEPES, pH 7.4, 300 mM NaCl, 0.1% Cymal-6 and applied to a protein A-Sepharose column (previously washed in water and buffer and blocked with 10% BSA in buffer). The flowthrough was reapplied to the protein A column. An aliquot of the final flowthrough was diluted appropriately and applied to a white 96-well plate at a volume of 50 μ l per well. The ELISA was performed as described above. The rest of the final flowthrough was concentrated and then characterized in additional experiments.

Cell surface Env immunoprecipitation. HIV-1_{AD8} Env(+) Δ 712 glycoproteins expressed on the surfaces of HOS cells and HEK 293T cells were incubated with approximately 1 μ g of anti-Env antibody in a total volume of 5 ml of 20 mM HEPES, pH 7.4, 300 mM NaCl, 2% BSA for 1 h at 37°C. Then, the cells were washed extensively with PBS and lysed with PBS, 1% Cymal-5, pH 7.4. Aliquots of 15 to 20 μ l of protein A-Sepharose beads (Life Technologies or Sigma) were blocked in blocking buffer (20 mM Tris HCl/HEPES, pH 7.4, 300 mM NaCl, 2 to 10% BSA) and then equilibrated in 20 mM Tris HCl/HEPES, pH 7.4, 300 mM NaCl, 0.1% Cymal-6. The beads and Env-Ab complexes were incubated for 1 h at room temperature and then vortexed and spun down. The supernatant was withdrawn, and the proteins bound to the beads were analyzed by SDS-PAGE. The gels were subjected to Western blotting with a rabbit anti-gp120 polyclonal serum.

Soluble Env immunoprecipitation. Purified HIV-1_{BG505} sgp140 SOSIP.664 trimers were incubated with a 2-fold molar concentration of antibodies (2G12, VRC01, VRC03, PG9, 19b, 902090, PGT128, 17b, PGT151, and a human IgG control) for 1 h at room temperature. Protein G-Sepharose beads were added to the reaction mixture, and the mixture was rotated at room temperature for another hour. The beads were pelleted by low-speed centrifugation and washed six times with PBS. Proteins bound to the beads were analyzed by SDS-PAGE and Western blotting with a rabbit anti-gp120 polyclonal serum.

Microscale thermophoresis. Ligand binding to purified Envs was analyzed by microscale thermophoresis on a Monolith NT.115 (Nanotemper Technologies) (92). The glutaraldehyde-cross-linked Envs were fluorescent, precluding the need for labeling with an additional fluorophore. The binding of the PG9 antibody to the cross-linked Envs was tested using antibody concentrations ranging from 0.061 nM to 2 μ M in 20 mM Tris-HCl, pH 7.5, 300 mM NaCl to detect aggregation propensity in solution. The glutaraldehyde-cross-linked Env was added to a final constant concentration of 20 nM, followed by a 1-h incubation at room temperature, and ligands (2G12, VRC01, sCD4, PG9, 4e10, and 17b) were titrated up to 4 μ M. The Env concentration was chosen so that the observed fluorescence was approximately 1,000 U at 50% LED power. The samples were loaded into Monolith capillaries and measured by standard protocols of temperature shifts using a Monolith NT.115 (NanoTemper), as described by the manufacturer. The changes in the fluorescent thermophoresis signal were plotted against the concentration of the serially diluted antibody, and dissociation constant (K_d) values were determined using NanoTemper analysis software.

Hydrodynamic radius calculation. The dynamic responses of the glutaraldehyde-cross-linked HIV-1_{AD8} Env(+) Δ 712 and Env(-) Δ 712 glycoproteins immobilized on a DNA lever chip were used to estimate the hydrodynamic radii of the trimers (93, 94). The biochip consisted of a glass substrate with eight holes (1-mm diameter), which served as inlets and outlets for four flow channels. Thiolated 90-mer oligonucleotides were grafted to gold electrodes and single-strand labeled with Cy3. Env glycoproteins (in 10 mM HEPES, 300 mM NaCl, 0.05% Cymal-6) were covalently coupled to a reactive (non-Cy3-labeled) strand via hydrazine-aldehyde chemistry. Fluorescent light from the Cy3-labeled DNA levers was excited by a green LED (517 nm). Emitted Cy3 fluorescence (570 nm) was detected with a photomultiplier (Hamamatsu). Alternating voltage square-wave signals were used to actuate the DNA molecules and to simultaneously trigger an event timer of a single photon at 32-ns time resolution. The switching potentials for the measurements were chosen to be close to the plateau of the fluorescence response (indicating completely lying and completely standing DNAs). The applied voltage amplitude was between 0.6 V and 1 V, and the frequency of the applied square-wave potential was 10 kHz, which ensured that sufficient time was available for the DNA to stand up and lie down for each measurement. Dynamic-response values were calculated from the normalized fluorescence signal and fitted into a theoretical Lollipop model for DNA behavioral movement, from which an R_h was determined. In the Lollipop model of the DNA-protein conjugate attached to the chip, the protein is treated as a potentially

charged sphere on top of a charged DNA rod. All experiments were carried out on a DRX 2400 instrument (Dynamic Sensors, Munich, Germany).

Negative-stain EM. The purified Envs (2 μ l of a 50- μ g/ml solution in Tris-buffered saline, 0.1% Cymal-6) were adsorbed to a glow-discharged carbon film grid, stained with 2% uranyl acetate, and imaged on an FEI Tecnai F20 transmission electron microscope with a 4,000-by-4,000 charge-coupled-device (CCD) camera (120 keV; $\times 80,000$ magnification; 20 electrons/ $\text{\AA}^2$).

The following numbers of particles were collected and analyzed for each Env glycoprotein: $\sim 50,000$ for HIV-1_{AD8} Env(+) $\Delta 712$, $\sim 20,000$ for HIV-1_{AD8} Env(−) $\Delta 712$, $\sim 10,000$ for HIV-1_{AD8} sgp140 SOSIP.664, and $\sim 10,000$ for HIV-1_{BG505} sgp140 SOSIP.664. Particle images were selected from the micrographs and assembled into class averages using common-lines methods in EMAN2 in a reference-free manner (122). Trimeric symmetry was imposed during the reconstruction/refinement. HIV-1_{AD8} Env(−) $\Delta 712$ failed to converge into a three-dimensional (3D) structure.

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