Promoting Trimerization of Soluble Human Immunodeficiency Virus Type 1 (HIV-1) Env through the Use of HIV-1/Simian Immunodeficiency Virus Chimeras

Rob J. Center,^{1*} Jacob Lebowitz,² Richard D. Leapman,² and Bernard Moss^{1*}

Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases,¹ and Division of Bioengineering and Physical Science, Office of Research Services,² National Institutes of Health, Bethesda, Maryland 20892

Received 11 July 2003/Accepted 14 November 2003

The envelope proteins (Env) of human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV) form homo-oligomers in the endoplasmic reticulum. The oligomeric structure of Env is maintained, but is less stable, after cleavage in a Golgi compartment and transport to the surface of infected cells. Functional, virion-associated HIV-1 and SIV Env have an almost exclusively trimeric structure. In addition, a soluble form of SIV Env (gp140) forms a nearly homogeneous population of trimers. Here, we describe the oligomeric structure of soluble, uncleaved HIV-1 gp140 and modifications that promote a stable trimeric structure. Biochemical and biophysical analyses, including sedimentation equilibrium and scanning transmission electron microscopy, revealed that unmodified HIV-1 gp140 purified as a heterogeneous range of oligomeric species, including dimers and aggregates. Deletion of the V2 domain alone or, especially, both the V1 and V2 domains reduced dimer formation but promoted aggregation rather than trimerization. Expressing gp140 with mannose-only oligosaccharides did not eliminate heterogeneity. Replacement of the entire gp41 segment of HIV-1 gp140 or just the N-terminal half (85 amino acids) of this segment with the corresponding region of SIV was sufficient to confer efficient trimerization for gp140 derived from clade B and C isolates. Importantly, the relatively small segment of the HIV Env replaced by SIV sequences contains no known targets of neutralizing antibody. The soluble trimeric form of HIV-1 Env should prove useful for assessment of antigenic structure and immunogenicity.

The human immunodeficiency virus type 1 (HIV-1) envelope protein (Env) is synthesized as a precursor molecule, gp160, which is processed via the same cellular pathway as other cell surface integral membrane proteins. Major processing steps in the endoplasmic reticulum include extensive glycosylation, disulfide bond formation, and oligomerization (21). Cleavage in the Golgi complex produces gp120 and the membrane-anchored gp41, which remain associated by noncovalent interactions. Complexes of gp120 and gp41 are transported to the cell surface, where incorporation into budding virions occurs. The env complex is indispensable for viral infectivity; gp120 interacts with the target cell receptors CD4 and one of the chemokine receptors (most often CCR5 or CXCR4), triggering conformational changes that culminate in gp41 fusion peptide insertion into the target cell membrane and the fusion of this membrane with that of the infected cell or virion (reviewed in reference 22).

Env is the only viral protein to protrude beyond the virion membrane, and it is the major viral target of the host humoral immune response. The oligomeric structure of env modulates antigenicity, presumably by reducing the exposure of epitopes close to contact sites between protomers and/or by directly altering epitope conformation. The ability of antibody to neutralize virus is better predicted by a capacity to bind to oligo-

meric Env than to monomeric Env (25, 26, 35). Because virion-associated HIV-1 Env is trimeric (9), it would be desirable for an env immunogen designed to elicit neutralizing antibodies to also have a trimeric structure. To obtain soluble Env oligomers for testing as immunogens, recombinant techniques have been employed to express Env lacking the transmembrane domain and cytoplasmic tail (gp140). Since cleavage at the gp120-gp41 junction causes the oligomeric contacts between protomers to become labile, the cleavage sites of most gp140s studied are inactivated by mutagenesis. Uncleaved gp140 has been variously reported to form dimers and tetramers (18), trimers and dimers (11, 44), dimers, trimers, and tetramers (40), and mainly trimers (51) and to largely fail to form stable oligomers (48, 49). Cleaved gp140 with engineered disulfide linkages between the gp120 and gp41 subunits was reported to form mainly monomers or oligomers with reduced stability (3, 40). In the present study, we used biochemical and biophysical methods to analyze uncleaved HIV-1 gp140 proteins and confirmed the formation of nontrimeric species including dimers and aggregates (defined here as any oligomer of more than three protomers). We had previously found that simian immunodeficiency virus (SIV) gp140 formed a relatively homogeneous population of trimers (10). Through the use of HIV-1/SIV gp140 chimeras, we show here that replacement of the N-terminal half of the gp41 segment of HIV-1 gp140 with the corresponding region of SIV is sufficient to promote efficient trimerization.

MATERIALS AND METHODS

env expression, purification, and gel filtration. The recombinant vaccinia virus vBD5 (16) was used to express gp140 derived from the HIV-1_{JR-FL} (GenBank

* Corresponding author. Mailing address for B. Moss: Laboratory of Viral Diseases, National Institutes of Health, Building 4, Room 229, 9000 Rockville Pike, Bethesda, MD 20892. Phone: (301) 496-9869. Fax: (301) 480-1147. E-mail: bmoss@nih.gov. Mailing address for R. J. Center: Department of Microbiology and Immunology, University of Melbourne, Parkville, Victoria 3010, Australia. Phone: 613-8344-9779. Fax: 613-9347-1540. E-mail: rcenter@unimelb.edu.au.

accession number U63632). Recombinant vaccinia viruses expressing HIV-1 gp140 with deletion mutations and HIV-1/SIV gp140 chimeras were produced by standard recombinant techniques using the HIV_{JR-FL} Env-encoding plasmid pCB28 (5) and DNA extracted from the recombinant vaccinia virus vAE1 (23). DNA extracted and amplified from the vAE1 virus encoded gp140 derived from the SIV_{CP-MAC} isolate (28) except for the following amino acid differences: S559→L, L573→V, T575→K and I588→T. The amino acid numbering used here is based on the full-length HIV-1_{JR-FL} or SIV_{CP-MAC} Env sequence with the initial methionine of the signal peptide as 1. For all viruses, gp140 expression was under the control of a synthetic early-late vaccinia virus promoter (12). For env expression using recombinant vaccinia viruses, BS-C-1 cells (an African green monkey kidney cell line) were infected at a multiplicity of infection of 5 and overlaid with serum-free Opti-MEM (Gibco BRL). After 1.5 to 2 days, the supernatant was centrifuged to remove cellular debris and then adjusted to 0.2% Triton X-100 to inactivate vaccinia virus. Although the cleavage site was intact in the vaccinia virus-expressed gp140 molecules, subsequent analysis showed that most of the secreted protein was uncleaved. Cleavage site-negative HIV-1_{ADA} and SIV_{Mac32H} gp140 (13, 51) were expressed in stably transfected CHO-Lec3.2.8.1 cells, which yield mannose-only oligosaccharides, using serum-free Opti-MEM plus sodium butyrate (2 mM final concentration) to induce expression. All gp140s were purified from the supernatants using lentil lectin affinity chromatography, as previously described (7). Concentrated eluates were subjected to gel filtration chromatography using a 16/60 Superdex 200 column (Amersham Pharmacia Biotech AB) with phosphate-buffered saline as the buffer. A flow rate of 0.5 ml/min was used, and 1-ml fractions were collected. env generally comprised ≥90% of the total protein in the fractions analyzed, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining. Similar methods were used to express and purify unmodified and chimeric gp140 derived from clade C 93MW965 (GenBank accession number U08455).

Electrophoresis, immunoblotting and chemical cross-linking. Individual gel filtration fractions were subjected to SDS-PAGE (8% polyacrylamide) under reducing conditions and transferred to nitrocellulose membranes. After being blocked with 4% bovine serum albumin, the membranes were sequentially probed with a rabbit serum raised against HIV-1 gp140 (IIIB/LAI strain) or monoclonal antibodies and iodinated protein A. Signal was visualized and quantified by phosphor screen autoradiography using a scanner and ImageQuant software (Molecular Dynamics). Cross-linking was performed by incubating samples in the presence of ethylene glycol bis(succinimidylsuccinate) (EGS) (Pierce) at a final concentration of 5 mM for 30 min at room temperature, followed by quenching with a final concentration of 100 mM glycine prior to SDS-PAGE (5% polyacrylamide) and immunoblotting as above. Blue-native PAGE was performed as previously described (40) using 4 to 12% Bis-Tris gels (Invitrogen).

Sedimentation equilibrium and velocity. Sedimentation equilibrium and velocity analyses were performed using a Beckman-Coulter analytical eight-cell An-50 Ti rotor with an Optima XL-A/I analytical ultracentrifuge in the absorbance optical-scanning mode. Sedimentation equilibrium was used to determine the weight-average molecular weight of gp140 within individual gel filtration fractions that were concentrated approximately eightfold (final concentration, approximately 0.25 to 0.75 mg/ml) prior to analysis. Cells were loaded with volumes of 120 to 135 μ l of sample and measured at either 230 or 280 nm in an optical density range of approximately 0.2 to 0.4 absorbance unit (AU). Absorbance-versus-radial-position step scanning data at radial increments of 0.001 cm with 20 repeats were obtained at 10°C using three different rotor speeds between 5,000 and 9,000 rpm for each sample. A global nonlinear regression analysis was performed using the data analysis software package provided by Beckman-Coulter Instruments (version 4.0 and Microcal version 4.1). The partial specific volume for each gp140 species was calculated from the amino acid sequence and an estimated partial specific volume of 0.622 for the carbohydrate component based on an analysis of glycoproteins (29). Mass-spectral analysis of vaccinia virus-expressed SIV_{CP-MAC} and HIV-1_{JR-FL} gp120 (cleaved from gp140) produced mass values of 99 and 93 kDa, respectively (D. Sheeley and R. Center, unpublished data), allowing for the determination of the average carbohydrate mass for each potential N-linked glycosylation site (2.06 kDa for SIV and 1.739 kDa for HIV-1). These values were used to estimate the carbohydrate mass of each vaccinia virus-expressed gp140 species. For gp140 produced in CHO-Lec3.2.8.1 cells, the mass of oligosaccharides with five mannose groups was used to calculate the carbohydrate component, assuming utilization of all potential N-linked sites.

Boundary sedimentation velocity analysis was performed at 20°C with rotor speeds of 25,000 or 30,000 rpm and scanning at 230 nm. The latter experiments were carried out directly after the sedimentation equilibrium analysis by gently tilting the rotor until the contents of the cells were uniformly redistributed.

Sedimentation coefficient distribution analysis was performed as previously described (39) using Sedfit software. The data presented were subjected to maximum-entropy regularization (39). This statistical treatment produced distributions consistent with the raw data within 95% confidence limits. Maximum-entropy regularization combined with the inherently heterogeneous glycosylation (and therefore mass) of env protomers tends to merge closely spaced peaks.

STEM. Scanning transmission electron microscopy (STEM) was performed as previously described (10). Briefly, 5- μ l aliquots of gp140 and tobacco mosaic virus were sequentially applied to copper grid-supported carbon films. The grids were washed, plunge-frozen into liquid ethane, cryotransferred to an HB501 STEM (VG Scientific), and freeze-dried. Annular dark-field images were acquired digitally using an electron dose of approximately 10^3 e/nm² and an acquisition time of 100 s. Images were processed and quantified using the IMAGE program (available at <http://rsb.info.nih.gov/nih-image/>). Mass values were calibrated using tobacco mosaic virus particles contained in the same field as gp140.

RESULTS

HIV-1_{JR-FL} gp140 purifies as a heterogeneous range of oligomeric species. Purified HIV-1_{JR-FL} gp140 was initially analyzed by gel filtration, which separates proteins on the basis of molecular size. HIV-1_{JR-FL} gp140 resolved as a broad gel filtration peak, eluting mainly between fractions 45 and 58 (Fig. 1A). Immunoblotting of EGS-cross-linked fractions showed that essentially all HIV-1 gp140 had a lower electrophoretic mobility than the monomer (Fig. 1B), which had an apparent mass of slightly less than 160 kDa (based on SDS-PAGE in the absence of cross-linking [Fig. 1A inset]). The presence of multiple species was apparent from the cross-linking profile. Fractions 54 to 57 contained protein migrating to a position slightly above the 250-kDa standard. This band position was similar to that which we observed for dimeric gp120 (7), suggesting the presence of gp140 dimers. Fractions 50 to 54 contained gp140 migrating to a position well above that of the 250-kDa standard but below the top of the gel. The presence of very slowly SDS-PAGE-migrating cross-linked protein (fractions 46 to 51, close to the top of the gel) indicated the presence of some Env molecules with a larger number of protomers than the two apparent species described above. Immunoblotting of the non-cross-linked sample revealed some protein within fractions 65 to 69 with an SDS-PAGE migration pattern consistent with that of monomeric gp120 (data not shown).

STEM is a quantitative method for measuring mass based on the elastic scattering of electrons by atoms within individual molecules. The STEM-measured masses of 622 molecules within fraction 51 showed a broad and asymmetrical distribution with a mean value of 444 kDa (3.71 protomers) and a standard deviation of 107 kDa (Fig. 1C; Table 1), consistent with the presence of at least some oligomers with more than three protomers. Given that functional native Env is a trimer, complexes of more than three protomers are presumably non-specifically associated; they are referred to hereafter as aggregates. The masses of 434 molecules within fraction 56 yielded a relatively symmetrical distribution with a mean value of 280 kDa (2.34 protomers) and a standard deviation of 64 kDa (Fig. 1D; Table 1), consistent with a predominantly dimeric structure. Overall, it is clear that in contrast to the homogeneous trimeric structure of virion-derived SIV and HIV-1 Env and SIV gp140 (9, 10, 13), HIV-1_{JR-FL} gp140 forms a heterogeneous range of oligomeric species including dimers, trimers, and higher-mass aggregates.

Sedimentation coefficient distribution [$c(s)$] analysis of sed-

FIG. 1. Analysis of the oligomeric structure of HIV-1_{JR-FL} gp140. Lentil lectin affinity-purified gp140 was passed through a column of Superdex 200, and individual gel filtration fractions were analyzed using biochemical and biophysical methods. (A) Aliquots of gel filtration fractions were subjected to SDS-PAGE (8% polyacrylamide) and immunoblotted with an Env-specific antiserum and iodinated protein A. gp140 was quantified by phosphor screen autoradiography, and the results were plotted as a percentage of the total gp140-specific signal. The inset shows SDS-PAGE (10% polyacrylamide) of a pool of fractions 51 to 56 as revealed by Coomassie blue staining. (B) Aliquots of fractions were treated with the cross-linker EGS (5 mM final concentration), analyzed by SDS-PAGE (5% polyacrylamide) and immunoblotted as above. The bar indicates the electrophoretic mobility of a 250-kDa marker protein. (C) STEM-derived mass measurements of 622 individual oligomers within fraction 51. (D) STEM-derived mass measurements of 434 individual oligomers within fraction 56. Arrowheads in panels C and D indicate the expected mass of 360 kDa for trimeric gp140.

imentation velocity data was performed to obtain model-free information about the oligomeric state of the molecules in solution. The distributions of HIV-1_{JR-FL} gp140 fractions 51, 54, and 56 showed significant separation (Fig. 2A), consistent with differences in protomer number. Sedimentation equilibrium, unlike gel filtration, allows shape-independent mass determinations for Env (7). The data sets for sedimentation equilibrium absorbance versus radial position (Fig. 2B) were

analyzed by global nonlinear regression. The masses determined by this analysis, and the calculated numbers of protomers (in parentheses) are shown in Table 2. Protein from fractions 56 and 55 gave sedimentation equilibrium-derived mass values close to those expected for the dimer (2.26 and 2.40 protomers respectively). gp140 from fractions 49 and 51 gave sedimentation equilibrium-derived mass values equating to 4.85 and 3.84 protomers, respectively, indicating that some of the molecules within these fractions were aggregated. Fractions from intermediate positions within the profile (fractions 52 to 54) produced sedimentation equilibrium-derived mass values converting to 3.58 to 3.14 protomers, consistent with the presence of trimers. The fact that fraction 54 (3.14 protomers) contained two EGS-cross-linked species suggests that a substantial nontrimeric component is also present within these fractions.

HIV-1_{ADA} and SIV_{Mac32H} gp140 expressed with mannose-only oligosaccharides have different oligomeric profiles. Recent studies have suggested the utility of HIV-1 and SIV gp140 with mannose-only oligosaccharides for biochemical and structural studies (13, 51). We sought to determine if mannose-only

TABLE 1. Masses of gp140 species as determined by STEM^a

Source of gp140	Fraction no.	Mass (kDa)	No. of protomers ^b
HIV-1 _{JR-FL}	51	444	3.71
	56	280	2.34
H-S	51–52	361	3.07
	53–54	360	3.06
H-S.N	51–53	403	3.36

^a Number average.

^b The mass of one protomer was estimated by summing the protein mass deduced from the amino acid sequence and the carbohydrate mass (see Materials and Methods).

FIG. 2. Sedimentation velocity and equilibrium analysis of HIV-1_{JR-FL} gp140. (A) Differential sedimentation coefficient distributions, $c(s)$, calculated from sedimentation velocity experiments for fractions 56 (solid line), 51 (dashed line), and 54 (dash-dot line). (B) Sedimentation equilibrium concentration profiles of gel filtration fractions 51 (squares), 52 (circles), 53 (triangles), and 54 (diamonds). Solid lines show the best-fit distributions after global modeling of data obtained at three different rotor speeds. For clarity, only data obtained at 6,000 rpm are shown. All plots depicted were derived from measurements at 280 nm except for the fraction 51 plot, which was derived from measurement at 230 nm. Residuals of the fitted lines to the experimental data are displayed in the lower panel. OD, optical density.

HIV-1 and SIV gp140 showed the different degree of oligomeric heterogeneity that we observed for normally glycosylated gp140s derived from HIV-1 and SIV. HIV-1_{ADA} and SIV_{Mac32H} gp140 (13, 51) were expressed in stably transfected CHO-Lec3.2.8.1 cells (43), which have mutations blocking complex-oligosaccharide addition, resulting in all utilized N-linked glycosylation sites having oligosaccharides with five mannose residues (30). Purified HIV-1_{ADA} and SIV_{Mac32H} gp140s were subjected to gel filtration and compared. SIV_{Mac32H} gp140 resolved as a symmetrical and relatively sharp peak with elution mainly between 52 and 59 ml (Fig. 3A, dashed line and open circles). HIV-1_{ADA} gp140 resolved as a broader and asymmetrical peak between 48 and 61 ml (Fig. 3A,

TABLE 2. Masses of gp140 species as determined by sedimentation equilibrium^a

Source of gp140	Fraction no.	Mass (kDa)	No. of protomers ^b
HIV-1 _{JR-FL}	49	580.4	4.85
	51	459.5	3.84
	52	428.8	3.58
	53	402.8	3.37
	54	375.4	3.14
	55	286.7	2.40
HIV-1 _{ADA}	56	270.3	2.26
	51	424.2	3.86
	53	344.8	3.13
	56	305.0	2.77
HIV-1 _{JR-FL} ΔV2	59	253.3	2.30
	51	486.3	4.40
	52	445.2	4.03
	53	398.3	3.60
HIV-1 _{JR-FL} ΔV1/2	54	364.5	3.30
	56	297.7	2.69
H-S	48	634.5	6.42
	51	487.5	4.93
S-H	50	535.3	4.55
	51	442.2	3.76
	52	408.2	3.47
	53	407.6	3.46
	54	381.9	3.25
	55	358.0	3.04
H-S.N	52	394.5	3.14
	56	275.2	2.19
	57	267.6	2.13
	62	166.2	1.32
HIV-1 _{93MW965}	50	469.1	3.91
	51	416.6	3.47
	52	398.6	3.32
	53	370.7	3.09
	54	351.9	2.93
	55	337.2	2.81
H _{93MW965} -S.N	50	442.1	3.46
	51	417.4	3.27
	52	389.5	3.05
	53	349.7	2.74
	54	322.1	2.52
	55	306.0	2.40
	50	452.6	3.55
	51	417.0	3.27
	52	426.2	3.34
	53	420.4	3.30
	54	415.0	3.25

^a Weight average.

^b The mass of one protomer was estimated by summing the protein mass deduced from the amino acid sequence and the carbohydrate mass (see Materials and Methods).

solid line and solid squares), suggesting greater size heterogeneity. BN-PAGE has recently been successfully used to analyze gp140 (40) and has proved more effective than EGS cross-linking for revealing the oligomeric profile of HIV-1_{ADA} gp140. The range of apparent masses revealed by BN-PAGE (approximately 670 to 220 kDa) (Fig. 3B) and the range of

FIG. 3. Gel filtration analysis of HIV-1_{ADA} and SIV_{Mac32H} gp140 expressed in CHO-Lec3.2.8.1 cells. (A) Aliquots of gel filtration fractions were subjected to SDS-PAGE (8% polyacrylamide) and immunoblotted with an Env-specific antiserum and iodinated protein A. gp140 was quantified by phosphor screen autoradiography, and the results were plotted as a percentage of the total gp140-specific signal for HIV-1_{ADA} (solid squares and solid line) and SIV_{Mac32H} (open circles and dashed line). The inset shows SDS-PAGE (10% polyacrylamide) of a pool of fractions 51, 53, 56, and 59 of HIV-1_{ADA} gp140 as revealed by Coomassie blue staining. (B) BN-PAGE (4 to 12% polyacrylamide) of the indicated HIV-1_{ADA} gp140 fractions. The 440/220- and 670/335-kDa markers were the dimers and monomers of ferritin and thyroglobulin, respectively.

sedimentation equilibrium-derived protomer numbers (3.86 to 2.30 [Table 2]) for fractions 51, 53, 56, and 59 of HIV-1_{ADA} gp140 confirmed oligomeric heterogeneity. The presumed trimeric component (Fig. 3B, major band, fractions 53 and 56) was enriched in comparison to HIV-1_{JR-FL} gp140 expressed in B-SC-1 cells. Nevertheless, HIV-1_{ADA} gp140 is less homogeneous in terms of oligomeric structure than is SIV_{Mac32H} gp140.

Deletion of HIV-1 variable domains 1 and 2 reduces dimer formation and promotes aggregation. The second variable domain (V2) can mediate dimer formation between recombinant gp120 subunits (7). We hypothesized that the same contact site may mediate gp140 dimer formation and that its elimination would redirect this subset of gp140 molecules to a pool avail-

able for trimerization. We therefore expressed HIV-1_{JR-FL} gp140 lacking either the V2 domain (amino acids F156 to L190) (HIV-1_{JR-FL}ΔV2) or the entire first and second variable domains (amino acids K120 to Q200 replaced with a GAG tripeptide) (HIV-1_{JR-FL}ΔV1/2) and analyzed the oligomeric structure as before. Note that the reduction in protomer mass due to deletion of the V2 or V1 plus V2 loops means that the average number of protomers per molecule for a given fraction number will be larger than the comparable value for nondeleted gp140 for corresponding fractions. Comparison of the gel filtration profile (Fig. 4A) for HIV-1_{JR-FL}ΔV1/2 (open squares and dot-dash line) to that of nondeleted gp140 (solid squares and solid line) showed significant skewing to larger sizes, with lower percentages of protein present in fractions 54 to 57, which in the nondeleted protein contained predominantly dimers. SDS-PAGE of EGS-cross-linked samples revealed an absence of protein with a migration consistent with the dimer (close to the 250-kDa marker) and the presence of protein with very slow migration within fractions 47 to 52 (Fig. 4B). The sedimentation equilibrium-derived mass values for two peak fractions 48 and 51 convert to 6.42 and 4.93 protomers, respectively (Table 2), indicating that most HIV-1_{JR-FL}ΔV1/2 gp140 was in the form of aggregates.

Deletion of the V2 domain only (Fig. 4A, open circles and dashed line) had a less dramatic effect on oligomer formation. Less cross-linked protein running close to the 250-kDa marker was detected in comparison to the nondeleted form (compare Fig. 4C with Fig. 1B fractions 54 to 57), and the sedimentation equilibrium results indicated a range of 3.60 to 2.69 protomers per molecule within fractions 53 to 56 (Table 2), suggesting the presence of fewer dimers and more trimers. The values seen for peak fractions 51 and 52 (4.40 and 4.03 protomers, respectively) and the presence of a slowly migrating EGS-cross-linked species in fractions 48 to 52 indicated that considerable aggregate formation had occurred. It is evident from these results that deletion of the V1 and V2 domains and, to a much lesser extent, deletion of the V2 domain alone markedly reduces dimer formation and promotes aggregation without greatly enhancing trimerization.

Replacing the gp41 subunit of HIV-1_{JR-FL} gp140 with that of SIV reduces dimer formation and promotes trimerization. We previously demonstrated that unmodified SIV gp140 purified as a homogeneous population of trimers (10). We therefore sought to identify the SIV env domain responsible for this property and to use it to promote HIV-1 gp140 trimerization by using a domain exchange strategy. Previous studies have shown that the gp41 component of HIV-1 and HIV-2 (closely related to SIV) contained the major determinants of env oligomerization (8, 20, 36). We therefore genetically combined HIV-1_{JR-FL} gp120 (amino acids M1 to R502) and SIV gp41 (amino acids G528 to A687) to create a chimeric gp140 (H-S). In comparison to HIV-1_{JR-FL} gp140, H-S gp140 displayed a sharper and more symmetrical gel filtration peak (Fig. 5A, open diamonds and dashed line compared to solid squares and solid line). Fractions 52 to 56 contained what appeared to be one major EGS-cross-linked species (Fig. 5B) that was shown by sedimentation equilibrium results (Table 2) to be trimeric (range, 3.47 to 3.04 protomers for fractions 52 to 55). No H-S EGS-cross-linked dimers were detected. BN-PAGE (4 to 12% polyacrylamide) of a pool of fractions 50 to 54 (Fig. 5A inset)

FIG. 4. Gel filtration analysis of variable-loop deletion mutants of HIV-1_{JR-FL} gp140. (A) Aliquots of gel filtration fractions were subjected to SDS-PAGE (8% polyacrylamide) and immunoblotted with an Env-specific antiserum and iodinated protein A. gp140 was quantified by phosphor screen autoradiography, and the results were plotted as a percentage of the total gp140-specific signal for HIV-1_{JR-FL}ΔV2 (open circles and dashed line) and HIV-1_{JR-FL}ΔV1/2 (open squares and dot-dash line). Intact HIV-1_{JR-FL} gp140 (see Fig. 1) is also shown for comparison (closed squares and solid line). Aliquots of fractions of HIV-1_{JR-FL}ΔV1/2 gp140 (B) and HIV-1_{JR-FL}ΔV2 gp140 (C) were treated with the cross-linker EGS (5 mM final concentration) and analyzed by SDS-PAGE (5% polyacrylamide) and immunoblotted as above. The bars indicate the electrophoretic mobility of a 250-kDa marker protein.

confirmed the presence of one predominant species. A faint band below the major band (migrating to a position between the 440- and 220-kDa markers) suggested the presence of trace amounts of dimer. Fractions 45 to 51 contained a very slowly

migrating EGS-cross-linked species with high mass (for example, for fraction 50 the average number of protomers was 4.55), indicating that as with HIV-1_{JR-FL} gp140, some H-S gp140 was aggregated. The sedimentation velocity profiles of H-S fractions 50 to 55 displayed considerable overlap (Fig. 5D). The profiles for fractions 50 and 51 showed some skewing to a higher sedimentation coefficient. Together, these observations are consistent with the formation of trimers and some aggregates. STEM analysis of 420 individual molecules for a pool of fractions 51 and 52 (Fig. 5E; Table 1) and 515 molecules from a pool of fractions 53 and 54 (Table 1) yielded mass values of 361 kDa (standard deviation, 70 kDa) and 360 kDa (standard deviation, 76 kDa), respectively, which convert to 3.07 and 3.06 protomers. This confirmed the trimeric nature of the major species. We observed that a portion of individual H-S gp140 molecules visualized by STEM had a triangular or trilobed morphology (a montage of molecules observed in the pool of fractions 51 and 52 is shown in the Fig. 5E inset), which was very similar to that which we previously reported for SIV gp140 trimers and virion-derived SIV and HIV-1 Env trimers (9, 10). Overall, the strategy of replacing the HIV-1 gp41 subunit with that of SIV was successful in redirecting oligomer formation from dimerization to trimerization.

A reverse chimera (S-H) composed of SIV gp120 (M1 to R527) and HIV-1_{JR-FL} gp41 (A503 to K674) was also tested. S-H gp140 showed a strikingly different gel filtration profile (Fig. 5A, open circles, dot-dash line) from that of either H-S or HIV-1_{JR-FL} gp140, with a marked skewing toward smaller size. This reflected (i) a greater percentage of dimer, present in fractions 53 to 57 (Fig. 5C), with the average number of protomers in fractions 56 and 57 being 2.19 and 2.13, respectively (Table 2); and (ii) a separate peak between fractions 61 and 64. The gel filtration elution volume of this peak, which indicated a smaller size than the dimer, and the sedimentation equilibrium-derived mass value for fraction 62, equating to 1.32 protomers (Table 2), indicated that this peak was composed of monomeric gp140. Some gp120 derived from gp140 cleavage was also present in this peak, as revealed by SDS-PAGE (data not shown). Less aggregate formation was apparent with S-H gp140 than with HIV-1_{JR-FL}, as evidenced by less very slowly migrating EGS-cross-linked material (compare Fig. 5C to Fig. 1B).

The N-terminal half of the SIV gp41 subunit is sufficient to promote trimerization. Our overall aim was to obtain trimeric soluble HIV-1 for future assessment as an immunogen. Since the C-terminal part of the gp41 ectodomain contains the epitopes of several broadly neutralizing monoclonal antibodies (33, 45, 52, 53), we considered it desirable to include the HIV-1 sequence spanning these epitopes in a new chimera. We therefore replaced the 74 C-terminal-most amino acids of H-S (SIV-derived amino acids, CP-MAC numbering from A614 to A687) with the corresponding sequence of HIV-1_{JR-FL} (amino acids S590 to K674) to create the chimera H-S.N. The increase in binding of the CD4-induced monoclonal antibody 17b in the presence of CD4 demonstrates that both the CD4 binding site and the conformationally sensitive 17b epitope are intact in H-S.N gp140 (Fig. 6A, lower inset). This suggests that the presence of the SIV gp41-derived sequence has not compromised the folding or function of H-S.N gp140. As with the H-S chimera, H-S.N gp140 revealed a sharper and more symmet-

FIG. 5. Analysis of the oligomeric structure of HIV-1/SIV gp140 chimeras. (A) Aliquots of gel filtration fractions were subjected to SDS-PAGE (8% polyacrylamide) and immunoblotted with an Env-specific antiserum (for H-S) or the monoclonal antibodies 36D5 (anti-SIV gp120) (23) and D50 (anti-HIV-1 gp41) (17) (for S-H) and iodinated protein A. gp140 was quantified by phosphor screen autoradiography, and the results were plotted as a percentage of the total gp140-specific signal for H-S (open diamonds and dashed line) and S-H (open circles and dot-dash line). Nonchimeric HIV-1_{JR-FL} gp140 (see Fig. 1) is also shown for comparison (solid squares and solid line). The inset shows BN-PAGE (4 to 12% polyacrylamide) of a pool of fractions 50 to 54 of H-S gp140 revealed by Coomassie blue staining. The upper and lower bars show the positions of the 440- and 220-kDa marker proteins, respectively. (B and C) Aliquots of fractions of H-S (B) and S-H (C) were treated with the cross-linker EGS (5 mM final concentration), analyzed by SDS-PAGE (5% polyacrylamide), and immunoblotted as above. The bars indicate the electrophoretic mobility of a 250-kDa marker protein. (D) Differential sedimentation coefficient distributions, $c(s)$, calculated from sedimentation velocity experiments for the indicated fractions of H-S. (E) STEM-derived mass measurements of 420 individual oligomers within a pool of fractions 51 and 52 of H-S. The inset shows a montage of STEM images that displayed a triangular or trilobed morphology. OD, optical density. Bar, 40 nm.

rical gel filtration peak than did HIV-1_{JR-FL} gp140 (Fig. 6A, open circles and dotted line compared to solid squares and solid line). The EGS-cross-linking profile indicated the presence of one predominant oligomeric species (Fig. 6B). This finding was supported by the substantial overlap of the sedimentation velocity profiles for fractions 50 to 55 (Fig. 6C). The sedimentation equilibrium results (Table 2) indicated that this species was a trimer, with a range for fractions 51 to 55 of 3.47 to 2.81 protomers. BN-PAGE (4 to 12% polyacrylamide) of a

pool of fractions 50 to 54 (Fig. 6A upper inset) revealed the presence of a single major band consistent with a mainly trimeric structure. A faint band below the major band (migrating to a position between the 440- and 220-kDa markers) suggested the presence of a small amount of dimer. Unexpectedly, less aggregation was detected for H-S.N gp140 than for H-S or HIV-1_{JR-FL} gp140 by EGS cross-linking, with less very slowly migrating protein present in fractions 48 to 51 (compare Fig. 6B to Fig. 5B and 1B). Mass measurement of STEM images of

FIG. 6. Analysis of the oligomeric structure of the HIV-1/SIV chimera H-S.N. (A) Aliquots of gel filtration fractions were subjected to SDS-PAGE (8% polyacrylamide) and immunoblotted with an Env-specific antiserum and iodinated protein A. gp140 was quantified by phosphor screen autoradiography, and the results were plotted as a percentage of the total gp140-specific signal for H-S.N. (open circles and dotted line). Nonchimeric HIV-1_{JR-FL} gp140 (see Fig. 1) is also shown for comparison (solid squares and solid line). The upper inset shows BN-PAGE (4 to 12% polyacrylamide) of a pool of fractions 50 to 54 of H-S.N. gp140 revealed by Coomassie blue staining. The upper and lower bars show the positions of the 440- and 220-kDa marker proteins, respectively. The lower inset shows the effect of the presence (+) or absence (-) of an excess of soluble four-domain CD4 on the immunoprecipitation of radiolabeled H-S.N. gp140 by monoclonal antibody 17b. (B) Aliquots of gel filtration fractions of H-S.N. gp140 were treated with the cross-linker EGS (5 mM final concentration), analyzed by SDS-PAGE (5% polyacrylamide), and immunoblotted as above. The bar indicates the electrophoretic mobility of a 250-kDa-marker protein. (C) Differential sedimentation coefficient distributions, $c(s)$, calculated from sedimentation velocity experiments for the indicated gel filtration fractions of H-S.N. gp140. (D) STEM-derived mass measurements of 265 individual oligomers within a pool of fractions 51 to 53 of H-S.N. gp140. (E) A montage of STEM images which displayed a triangular or trilobed morphology. OD, optical density. Bar, 40 nm.

265 individual molecules within peak fractions 51 to 53 (Fig. 6D) yielded a mean mass of 403 kDa (3.36 protomers) with a standard deviation of 85 kDa (Table 1), confirming the predominance of the trimeric species. As with H-S gp140, some of the STEM images of H-S.N. gp140 showed a triangular or trilobed morphology (Fig. 6E). The N-terminal half of SIV gp41 is therefore sufficient to confer efficient gp140 trimerization.

To test the general applicability of the strategy used to enhance the trimerization of H-S.N. gp140, we compared an unmodified gp140 derived from an HIV-1 clade C primary viral isolate 93MW965 and the equivalent of the H-S.N. chimera in the HIV-1_{93MW965} background. In this chimera, the N-terminal sequence of the SIV_{CP-MAC} gp41 (amino acids G528 to A610) replaces the corresponding HIV-1_{93MW965} gp41 sequence (amino acids A501 to M584). The gel filtration peak for

FIG. 7. Gel filtration analysis of gp140 and the HIV-1/SIV H-S.N chimera derived from clade C HIV-1_{93MW965}. (A) Aliquots of gel filtration fractions were subjected to SDS-PAGE (8% polyacrylamide) and immunoblotted with an Env-specific antiserum and iodinated protein A. gp140 was quantified by phosphor screen autoradiography, and the results were plotted as a percentage of the total gp140-specific signal for HIV-1_{93MW965} gp140 (solid squares and solid line) and the clade C H-S.N chimera (open circles and dashed line). Aliquots of fractions of HIV-1_{93MW965} gp140 (B) and the clade C H-S.N gp140 (C) were treated with the cross-linker EGS (5 mM final concentration), analyzed by SDS-PAGE (5% polyacrylamide) and immunoblotted as above. The bars indicate the electrophoretic mobility of a 250-kDa marker protein.

HIV-1_{93MW965} gp140 is sharper and more symmetrical than that of JR-FL gp140 (Fig. 7A compared to Fig. 1A, solid squares and solid line in both), indicating less oligomeric heterogeneity. The EGS cross-linking profile which revealed the presence of two oligomeric species (Fig. 7B), one with more rapid electrophoretic migration (seen in fractions 53 to 56) and one with slower migration (seen mainly in fractions 50 to 54), but less of the very slowly migrating protein aggregates seen for

JR-FL gp140 (fractions 46 to 51 in Fig. 1B). The sedimentation equilibrium data are consistent with the presence mainly of trimers and dimers (range, 3.46 to 2.40 protomers for fractions 50 to 55). The gel filtration profile of the clade C H-S.N gp140 chimera is similar overall to that of the unmodified molecule but lacks the shoulder on the more slowly eluting side of the peak seen with HIV-1_{93MW965} gp140 (Fig. 7A, open circles and dotted line compared to solid squares and solid line). The EGS-cross-linking profile reveals the presence of one predominant oligomeric species, which was shown by sedimentation equilibrium analysis to be composed of three protomers (range, 3.55 to 3.25 for fractions 50 to 54). The ability of the N-terminal half of SIV gp41 to confer efficient gp140 trimerization is therefore applicable to both the divergent strains tested (clades B and C).

DISCUSSION

We show here that gp140s derived from the clade B primary isolate HIV-1_{JR-FL} and the clade C primary isolate HIV-1_{93MW965} have a propensity to form nontrimeric oligomers including dimers and aggregates. This oligomeric heterogeneity was also observed for gp140s derived from a T-cell-line-adapted clade B isolate (the BH8 clone of IIIB/LAI) and primary isolates of clades A and D (J. Lebowitz and R. Center, unpublished data). An attempt to enhance trimerization by expressing HIV-1_{JR-FL} gp140 at a lower temperature (32°C), which has been shown to facilitate protein folding in some systems, did not significantly alter the oligomeric profile (data not shown). These results are in contrast to the virion-associated Env of both HIV-1 and SIV and to SIV gp140, which display a relatively homogeneous trimeric structure. Previous studies showed that recombinant, cell-associated HIV-1 gp160 also formed a mixture of oligomeric species including dimers (15, 19), suggesting that the heterogeneity we observed for HIV-1 gp140 may be a general property of HIV-1 Env. If so, it is likely that a cellular quality control mechanism prevents nontrimeric Env from being incorporated into virions, perhaps by preventing egress to the cell surface. The greater level of oligomeric heterogeneity of HIV-1 in comparison to SIV was also shown for molecules expressed in the absence of complex-type oligosaccharides. Enrichment of the trimeric component of HIV-1_{ADA} mannose-only gp140 in comparison to HIV-1_{JR-FL} gp140 with complex-type oligosaccharides present was observed. This may be due to strain-related differences rather than to differences in carbohydrate type, since expression of HIV-1_{JR-FL} gp140 using the recombinant vaccinia virus described in this study in the cell line producing mannose-only oligosaccharides (CHO-Lec3.2.8.1) did not result in an enrichment of the trimeric component (Lebowitz and Center, unpublished). Based on the ability of the HIV-1_{IIIB/LAI} V2 loop to mediate gp120 dimer formation (7), we hypothesized that deletion of this domain might block gp140 dimerization and enhance trimerization. Deletion of both the V1 and V2 loops did block gp140 dimer formation; however, most of this protein was aggregated rather than trimeric. Deletion of the V2 loop alone had a more modest effect, with a reduction of dimerization observed, but again trimer formation was not obviously enhanced. It has been reported that deletions in this region of env expose underlying conserved, potentially neutralizing

epitopes and that such deletions may therefore be advantageous in potential immunogens (1, 6, 14, 27, 37, 41, 42, 47). If the pronounced increase in JR-FL gp140 aggregation with deletion of the entire V1-V2 loop structure observed here is a general rather than strain-specific effect, this particular modification may be undesirable from a structural standpoint for potential immunogens, at least in the context of soluble gp140.

Analysis of a gp140 chimera composed of the SIV gp120 and HIV-1_{JR-FL} gp41 segments revealed a strong propensity of the HIV-1 gp41 domain for dimer formation. Furthermore, a significant fraction of the gp140 of this chimera failed to oligomerize altogether, suggesting that the oligomeric interface may be less stable. Conversely, replacement of either all or just the N-terminal half of the gp41 segment of HIV-1 gp140 with the homologous region of SIV was sufficient to block dimer formation and promote trimerization. These results are consistent with previous studies demonstrating the role of the N-terminal section of gp41 in oligomerization (8, 20, 36) and suggest that this region plays a role not only in oligomer formation per se but also in the type of oligomers produced. The reason for the apparent differences in the gp41 oligomerization domain of HIV-1 and SIV is not immediately clear. It may be speculated that the apparently reduced stability of the oligomeric contacts in pre-receptor-activated HIV-1 env trimers may facilitate the triggering of the conformational changes induced by receptor binding, and therefore may enhance fusogenicity, at the expense of efficient trimer formation. Such an explanation would imply that the separate evolutionary courses of the two viruses favored such changes in HIV-1 but not SIV.

The gp140 chimeras comprising either all or just the N-terminal half of the SIV gp41 segment in an HIV-1_{JR-FL} background showed less aggregate formation than did unaltered HIV-1 gp140. The fact that the reverse chimera (SIV gp120, HIV-1_{JR-FL} gp41) also showed less aggregate formation suggests that the HIV-1 gp41 segment does not directly induce aggregation. If aggregates form from the nontrimeric pool of molecules, reducing this pool by increasing the efficiency of trimerization (by replacing the HIV-1 oligomerization domain with that of SIV) may concomitantly reduce aggregate formation. Unexpectedly, the gp140 chimera H-S.N with the N-terminal half of SIV gp41 in an HIV-1_{JR-FL} background showed less aggregate formation than did the H-S chimera where the entire gp41 domain was exchanged with SIV. It has been suggested that changes which reduce the affinity of interaction between N- and C-terminal alpha-helical regions of gp41 can block the formation of a receptor-activated conformation and therefore may stabilize the pre-receptor-activated (native) Env trimer (31, 38). It may be predicted that the affinity between the N-terminal helix of SIV and the C-terminal helix of HIV-1 (as in H-S.N) is weaker than the affinity between the same-virus-type helices (all HIV-1 or all SIV), as evidenced by the finding that a peptide analogue of the HIV-1 C-terminal helix which potentially blocked HIV-1 infectivity was required at a much higher concentration to inhibit the infectivity of HIV-2 (a virus closely related to SIV) (46). This weaker affinity between helices in H-S.N gp140 may therefore promote gp140 trimerization and reduce the pool of nonnative gp140 available for aggregate formation. Alternatively, interaction between the N- and C-terminal helices may directly result in aggregation.

The potentially advantageous structural properties of the H-S.N chimera (efficient trimerization, reduced aggregation) are fortuitous, since this construct includes several broadly neutralizing HIV-1 epitopes in the C-terminal segment of gp41 (33, 45, 52, 53) that are absent in the H-S chimera. The non-HIV-1 segments of the H-S.N chimeras (the N-terminal half of gp41) includes the fusion peptide and the N-terminal alpha-helical regions, which have been found to be poorly immunogenic (2, 17), and part of the immunodominant epitope, which generally elicits nonneutralizing antibodies. Other approaches used to promote HIV-1 gp140 trimerization include the addition of heterologous GCN4- or fibrinogen-based trimerization motifs to the C terminus of gp140 (48–50). The use of the more closely related SIV motif may allow closer mimicking of the authentic HIV-1 Env trimer. We are currently assessing the antigenicity and immunogenicity of the clade B and C H-S.N gp140 constructs, as well as further defining the minimal SIV sequence required to confer efficient trimerization. There is much current interest in the possible use of soluble env analogues to elicit neutralizing-antibody responses. Env modifications aiming at improved elicitation of neutralizing antibodies include deletions of variable loops to expose underlying conserved epitopes (1, 14, 27, 32, 37), mutations to enhance proteolytic cleavage and introduce disulfide bonds capable of stabilizing gp120-gp41 complexes (3, 4, 40), chemical coupling of env and CD4 to stabilize the CD4-induced conformation (24), deletion of the gp41 fusion peptide and the interhelical region to stabilize the pre-receptor-activated conformation (11), and hyperglycosylation to focus the humoral immune response toward known broadly neutralizing epitopes (34). The strategy employed with the H-S.N chimera described in the present study offers an Env format allowing such modifications to be tested in a trimeric context.

ACKNOWLEDGMENTS

We thank R. Doms for providing recombinant vaccinia viruses, G. Stubbs for providing tobacco mosaic virus, J. Hoxie for providing monoclonal antibody 36D5, E. Reinherz for providing stably transfected CHO-Lec3.2.8.1 cell lines expressing HIV-1_{ADA} and SIV_{Mac32H} gp140, D. Sheeley for performing mass spectral analysis, C. Broder for providing plasmid pCB28, and N. Cooper for providing cells. The HIV-1_{93MW965} molecular clone was provided by B. Hahn through WHO-UNAIDS and the NIH AIDS Research and Reference Reagent Program. Monoclonal antibody 17b was provided by J. Robinson through the NIH AIDS Research and Reference Reagent Program. We also thank P. Schuck and P. Earl for helpful discussions.

This work was supported in part by a National Institutes of Health Intramural AIDS Targeted Antiviral Program grant.

REFERENCES

1. Barnett, S. W., S. Lu, I. Srivastava, S. Cherpelis, A. Gettie, J. Blanchard, S. Wang, I. Mboudjeka, L. Leung, Y. Lian, A. Fong, C. Buckner, A. Ly, S. Hilt, J. Ulmer, C. T. Wild, J. R. Mascola, and L. Stamatatos. 2001. The ability of an oligomeric human immunodeficiency virus type 1 (HIV-1) envelope antigen to elicit neutralizing antibodies against primary HIV-1 isolates is improved following partial deletion of the second hypervariable region. *J. Virol.* 75:5526–5540.
2. Binley, J. M., H. J. Ditzel, C. F. Barbas III, N. Sullivan, J. Sodroski, P. W. H. I. Parren, and D. R. Burton. 1996. Human antibody responses to HIV type 1 glycoprotein 41 cloned in phage display libraries suggest three major epitopes are recognized and give evidence for conserved antibody motifs in antigen binding. *AIDS Res. Hum. Retrovir.* 12:911–924.
3. Binley, J. M., R. W. Sanders, B. Clas, N. Schuelke, A. Master, Y. Guo, F. Kajumo, D. J. Anselma, P. J. Maddon, W. C. Olson, and J. P. Moore. 2000. A recombinant human immunodeficiency virus type 1 envelope glycoprotein complex stabilized by an intermolecular disulfide bond between the gp120

- and gp41 subunits is an antigenic mimic of the trimeric virion-associated structure. *J. Virol.* **74**:627–643.
4. Binley, J. M., R. W. Sanders, A. Master, C. S. Cavanaugh, C. L. Wiley, L. Schiffrer, B. Travis, S. Kuhmann, D. R. Burton, S.-L. Hu, W. C. Olson, and J. P. Moore. 2002. Enhancing the proteolytic maturation of human immunodeficiency virus type 1 envelope glycoproteins. *J. Virol.* **76**:2606–2616.
 5. Broder, C. C., and E. A. Berger. 1995. Fusogenic selectivity of the envelope glycoprotein is a major determinant of human immunodeficiency virus type 1 tropism for CD4⁺ T-cell lines vs. primary macrophages. *Proc. Natl. Acad. Sci. USA* **92**:9004–9008.
 6. Cao, J., N. Sullivan, E. Desjardins, C. Parolin, J. Robinson, R. Wyatt, and J. Sodroski. 1997. Replication and neutralization of human immunodeficiency virus type 1 lacking the V1 and V2 variable loops of the gp120 envelope glycoprotein. *J. Virol.* **71**:9808–9812.
 7. Center, R. J., P. L. Earl, J. Lebowitz, P. Schuck, and B. Moss. 2000. The human immunodeficiency virus type 1 gp120 V2 domain mediates gp41-independent intersubunit contacts. *J. Virol.* **74**:4448–4455.
 8. Center, R. J., B. E. Kemp, and P. Pombourios. 1997. Human immunodeficiency virus type 1 and 2 envelope glycoproteins oligomerize through conserved sequences. *J. Virol.* **71**:5706–5711.
 9. Center, R. J., R. D. Leapman, J. Lebowitz, L. O. Arthur, P. L. Earl, and B. Moss. 2002. Oligomeric structure of the human immunodeficiency virus type 1 envelope protein on the virion surface. *J. Virol.* **76**:7863–7867.
 10. Center, R. J., P. Schuck, R. D. Leapman, L. O. Arthur, P. L. Earl, B. Moss, and J. Lebowitz. 2001. Oligomeric structure of virion-associated and soluble forms of the simian immunodeficiency virus envelope protein in the prefusion activated conformation. *Proc. Natl. Acad. Sci. USA* **98**:14877–14882.
 11. Chakrabarti, B. K., W.-P. Kong, B.-Y. Wu, Z.-Y. Yang, J. Friberg, X. Ling, S. R. King, D. C. Montefiori, and G. J. Nabel. 2002. Modifications of the human immunodeficiency virus envelope glycoprotein enhance immunogenicity for genetic immunization. *J. Virol.* **76**:5357–5368.
 12. Chakrabarti, S., J. R. Sisler, and B. Moss. 1997. Compact, synthetic, vaccinia virus early/late promoter for protein expression. *BioTechniques* **23**:1094–1097.
 13. Chen, B., G. Zhou, M. Kim, Y. Chishti, R. E. Hussey, B. Ely, J. J. Skehel, E. L. Reinherz, S. C. Harrison, and D. C. Wiley. 2000. Expression, purification, and characterization of gp160e, the soluble, trimeric ectodomain of the simian immunodeficiency virus envelope glycoprotein, gp160. *J. Biol. Chem.* **275**:34946–34953.
 14. Cherpelis, S., I. Shrivastava, A. Gettie, X. Jin, D. D. Ho, S. W. Barnett, and L. Stamatatos. 2001. DNA vaccination with the human immunodeficiency virus type 1 SF162ΔV2 envelope elicits immune responses that offer partial protection from simian/human immunodeficiency virus infection to CD8⁺ T-cell-depleted rhesus macaques. *J. Virol.* **75**:1547–1550.
 15. Doms, R. W., P. L. Earl, and B. Moss. 1991. The assembly of the HIV-1 env glycoprotein into dimers and tetramers. *Adv. Exp. Med. Biol.* **300**:203–219.
 16. Doranz, B. J., S. S. W. Baik, and R. W. Doms. 1999. Use of a gp120 binding assay to dissect the requirements and kinetics of human immunodeficiency virus fusion events. *J. Virol.* **73**:10346–10358.
 17. Earl, P. L., C. C. Broder, R. W. Doms, and B. Moss. 1997. Epitope map of human immunodeficiency virus type 1 gp41 derived from 47 monoclonal antibodies produced by immunization with oligomeric envelope protein. *J. Virol.* **71**:2674–2684.
 18. Earl, P. L., C. C. Broder, D. Long, S. A. Lee, J. Peterson, S. Chakrabarti, R. W. Doms, and B. Moss. 1994. Native oligomeric human immunodeficiency virus type 1 envelope glycoprotein elicits diverse monoclonal antibody reactivities. *J. Virol.* **68**:3015–3026.
 19. Earl, P. L., R. W. Doms, and B. Moss. 1990. Oligomeric structure of the human immunodeficiency virus type 1 envelope glycoprotein. *Proc. Natl. Acad. Sci. USA* **87**:648–652.
 20. Earl, P. L., and B. Moss. 1993. Mutational analysis of the assembly domain of the HIV-1 envelope glycoprotein. *AIDS Res. Hum. Retrovir.* **9**:589–594.
 21. Earl, P. L., B. Moss, and R. W. Doms. 1991. Folding, interaction with GRP78-BiP, assembly, and transport of the human immunodeficiency virus type 1 envelope protein. *J. Virol.* **65**:2047–2055.
 22. Eckert, D. M., and P. S. Kim. 2001. Mechanisms of viral membrane fusion and its inhibition. *Annu. Rev. Biochem.* **70**:777–810.
 23. Edinger, A. L., M. Ahuja, T. Sung, K. C. Baxter, B. Haggarty, R. W. Doms, and J. A. Hoxie. 2000. Characterization and epitope mapping of neutralizing monoclonal antibodies produced by immunization with oligomeric simian immunodeficiency virus envelope protein. *J. Virol.* **74**:7922–7935.
 24. Fouts, T., K. Godfrey, K. Bobb, D. Montefiori, C. V. Hanson, V. S. Kalyanaraman, A. DeVico, and R. Pal. 2002. Crosslinked HIV-1 envelope-CD4 receptor complexes elicit broadly cross-reactive neutralizing antibodies in rhesus macaques. *Proc. Natl. Acad. Sci. USA* **99**:11842–11847.
 25. Fouts, T. R., J. M. Binley, A. Trkola, J. E. Robinson, and J. P. Moore. 1997. Neutralization of the human immunodeficiency virus type 1 primary isolate JR-FL by human monoclonal antibodies correlates with antibody binding to the oligomeric form of the envelope glycoprotein complex. *J. Virol.* **71**:2779–2785.
 26. Fouts, T. R., A. Trkola, M. S. Fung, and J. P. Moore. 1998. Interactions of polyclonal and monoclonal anti-glycoprotein 120 antibodies with oligomeric glycoprotein 120-glycoprotein 41 complexes of a primary HIV type 1 isolate: relationship to neutralization. *AIDS Res. Hum. Retrovir.* **14**:591–597.
 27. Kim, Y. B., D. P. Han, C. Cao, and M. W. Cho. 2003. Immunogenicity and ability of variable loop-deleted human immunodeficiency virus type 1 envelope glycoproteins to elicit neutralizing antibodies. *Virology* **305**:124–137.
 28. LaBranche, C. C., M. M. Sauter, B. S. Haggarty, P. J. Vance, J. Romano, T. K. Hart, P. J. Bugelski, and J. A. Hoxie. 1994. Biological, molecular, and structural analysis of a cytopathic variant from a molecularly cloned simian immunodeficiency virus. *J. Virol.* **68**:5509–5522. (Erratum, **68**:7665–7667.)
 29. Lewis, M. S., and R. P. Junghans. 2000. Ultracentrifugal analysis of molecular mass of glycoproteins of unknown or ill-defined carbohydrate composition. *Methods Enzymol.* **321**:136–149.
 30. Liu, J., A. G. D. Tse, H.-C. Chang, J.-H. Liu, J. Wang, R. E. Hussey, Y. Chishti, B. Rheinhold, R. Spoerl, S. G. Nathenson, J. C. Sacchettini, and E. L. Reinherz. 1996. Crystallization of a deglycosylated T cell receptor (TCR) complexed with an anti-TCR Fab fragment. *J. Biol. Chem.* **271**:33639–33646.
 31. Liu, J., S. Wang, J. A. Hoxie, C. C. LaBranche, and M. Lu. 2002. Mutations that destabilize the gp41 core are determinants for stabilizing the simian immunodeficiency virus-CPmac envelope glycoprotein complex. *J. Biol. Chem.* **277**:12891–12900.
 32. Lu, S., R. Wyatt, J. F. L. Richmond, F. Mustafa, S. Wang, J. Weng, D. C. Montefiori, J. Sodroski, and H. L. Robinson. 1998. Immunogenicity of DNA vaccines expressing human immunodeficiency virus type 1 envelope glycoprotein with and without deletions in the V1/2 and V3 regions. *AIDS Res. Hum. Retrovir.* **14**:151–155.
 33. Muster, T., F. Steindl, M. Pertscher, 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.
 34. Pantophlet, R., I. A. Wilson, and D. R. Burton. 2003. Hyperglycosylated mutants of human immunodeficiency virus (HIV) type 1 monomeric gp120 as novel antigens for HIV vaccine design. *J. Virol.* **77**:5889–5901.
 35. Parren, P. W. H. I., I. Mondor, D. Naniche, H. J. Ditzel, P. J. Klasse, D. R. Burton, and Q. J. Sattentau. 1998. Neutralization of human immunodeficiency virus type 1 by antibody to gp120 is determined primarily by occupancy of sites on the virion irrespective of epitope specificity. *J. Virol.* **72**:3512–3519.
 36. Pombourios, P., K. A. Wilson, R. J. Center, W. El Ahmar, and B. E. Kemp. 1997. Human immunodeficiency virus type 1 envelope glycoprotein oligomerization requires the gp41 amphipathic α -helical/leucine zipper-like sequence. *J. Virol.* **71**:2041–2049.
 37. Sanders, R. W., L. Schiffrer, A. Master, F. Kajumo, Y. Guo, T. Dragic, J. P. Moore, and J. M. Binley. 2000. Variable-loop-deleted variants of the human immunodeficiency virus type 1 envelope glycoprotein can be stabilized by an intermolecular disulfide bond between the gp120 and gp41 subunits. *J. Virol.* **74**:5091–5100.
 38. Sanders, R. W., M. Vesanan, N. Schuelke, A. Master, L. Schiffrer, R. Kalyanaraman, M. Paluch, B. Berkhout, P. J. Maddon, W. C. Olson, M. Lu, and J. P. Moore. 2002. Stabilization of the soluble, cleaved, trimeric form of the envelope glycoprotein complex of human immunodeficiency virus type 1. *J. Virol.* **76**:8875–8889.
 39. Schuck, P. 2000. Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and Lamm equation modeling. *Biophys. J.* **78**:1606–1619.
 40. Schülke, N., M. S. Vesanan, R. W. Sanders, P. Zhu, M. Lu, D. J. Anselma, A. R. Villa, P. W. H. I. Parren, J. M. Binley, K. H. Roux, P. J. Maddon, J. P. Moore, and W. C. Olson. 2002. Oligomeric and conformational properties of a proteolytically mature, disulfide-stabilized human immunodeficiency virus type 1 gp140 envelope glycoprotein. *J. Virol.* **76**:7760–7776.
 41. Srivastava, I. K., K. VanDorsten, L. Vojtech, S. W. Barnett, and L. Stamatatos. 2003. Changes in the immunogenic properties of soluble gp140 human immunodeficiency virus envelope constructs upon partial deletion of the second hypervariable region. *J. Virol.* **77**:2310–2320.
 42. Stamatatos, L., and C. Cheng-Mayer. 1998. An envelope modification that renders a primary, neutralization-resistant clade B human immunodeficiency virus type 1 isolate highly susceptible to neutralization by sera from other clades. *J. Virol.* **72**:7840–7845.
 43. Stanley, P. 1989. Chinese hamster ovary cell mutants with multiple glycosylation defects for production of glycoproteins with minimal carbohydrate heterogeneity. *Mol. Cell. Biol.* **9**:377–383.
 44. Staropoli, I., C. Chanel, M. Girard, and R. Altmeyer. 2000. Processing, stability, and receptor binding properties of oligomeric envelope glycoprotein from a primary HIV-1 isolate. *J. Biol. Chem.* **275**:35137–35145.
 45. Stiegler, G., R. Kunert, M. Pertscher, S. Wolbank, R. Voglauer, F. Steindl, and H. Katinger. 2001. A potent cross-clade neutralizing human monoclonal antibody against a novel epitope on gp41 of human immunodeficiency virus type 1. *AIDS Res. Hum. Retrovir.* **17**:1757–1765.
 46. Wild, C. T., D. C. Shugars, T. K. Greenwell, C. B. McDaniel, and T. J. Matthews. 1994. Peptides corresponding to a predictive α -helical domain of human immunodeficiency virus type 1 gp41 are potent inhibitors of virus infection. *Proc. Natl. Acad. Sci. USA* **91**:9770–9774.

47. Wyatt, R., N. Sullivan, M. Thali, H. Repke, D. Ho, J. Robinson, M. Posner, and J. Sodroski. 1993. Functional and immunologic characterization of human immunodeficiency virus type 1 envelope glycoproteins containing deletions of the major variable regions. *J. Virol.* **67**:4557–4565.
48. Yang, X., M. Farzan, R. Wyatt, and J. Sodroski. 2000. Characterization of stable, soluble trimers containing complete ectodomains of human immunodeficiency virus type 1 envelope glycoproteins. *J. Virol.* **74**:5716–5725.
49. 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.
50. Yang, X., J. Lee, E. M. Mahony, P. D. Kwong, R. Wyatt, and J. Sodroski. 2002. Highly stable trimers formed by human immunodeficiency virus type 1 envelope glycoproteins fused with the trimeric motif of T4 bacteriophage fibritin. *J. Virol.* **76**:4634–4642.
51. Zhang, C. W.-H., Y. Chishti, R. E. Hussey, and E. L. Reinherz. 2001. Expression, purification, and characterization of recombinant HIV gp140. The gp41 ectodomain of HIV or simian immunodeficiency virus is sufficient to maintain the retroviral envelope glycoprotein as a trimer. *J. Biol. Chem.* **276**:39577–39585.
52. Zwick, M. B., A. F. Labrijn, M. Wang, C. Spenlehauer, E. O. Saphire, J. M. Binley, J. P. Moore, G. Stiegler, H. Katinger, D. R. Burton, and P. W. H. I. Parren. 2001. Broadly neutralizing antibodies targeted to the membrane-proximal external region of human immunodeficiency virus type 1 glycoprotein gp41. *J. Virol.* **75**:10892–10905.
53. Zwick, M. B., M. Wang, P. Poignard, G. Stiegler, H. Katinger, D. R. Burton, and P. W. H. I. Parren. 2001. Neutralization synergy of human immunodeficiency virus type 1 primary isolates by cocktails of broadly neutralizing antibodies. *J. Virol.* **75**:12198–12208.