Purified, Proteolytically Mature HIV Type 1 SOSIP gp140 Envelope Trimers

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ABSTRACT

HIV type 1 (HIV-1) envelope is a noncovalent trimer of gp120–gp41 heterodimers, and its lability has hindered structural studies. SOSIP gp140 is a soluble, proteolytically mature form of the HIV-1 envelope wherein gp120–gp41 interactions are stabilized via a disulfide bond and gp41 contains an additional trimer-stabilizing point mutation. We describe the isolation of a substantially pure preparation of SOSIP gp140 trimers derived from KNH1144, a subtype A isolate. Following initial purification, the only significant contaminant was higher-order gp140 aggregates; however, 0.05% Tween 20 quantitatively converted these aggregates into trimers. The surfactant effect was rapid, dose dependent, and similarly effective for a subtype B SOSIP gp140. Surfactant-treated SOSIP gp140 retained favorable antigenicity and formed compact trimers 12–13 nm in size as determined by electron microscopy. This report provides the first description of homogeneous, cleaved HIV-1 envelope trimers. These proteins may be useful as vaccine immunogens and for studying structure–function relationships within the HIV-1 envelope glycoproteins.

INTRODUCTION

An estimated 40 million people were living with HIV worldwide at the end of 2005, with over 4 million newly infected cases in that year. Among the hardest hit areas is Sub-Saharan Africa, with over 25 million people living with HIV and about 10% dying of AIDS-related illnesses each year. Prophylactic measures, such as an HIV vaccine, have the potential to curtail the spread of HIV infection globally. Ideally, an HIV vaccine would protect against infection. One approach has been to target the HIV entry pathway, specifically focusing on the viral envelope spike.

The HIV-1 envelope (Env) glycoproteins are derived from a gp160 precursor protein, which is cleaved intracellularly by members of the furin family of endoproteases to yield a gp120 surface glycoprotein and a gp41 transmembrane glycoprotein. The fusogenic form of Env is a trimer of gp120–gp41 heterodimers that are associated via noncovalent interactions. HIV-1 gp120 binds CD4 and a coreceptor molecule on susceptible host cells and thereby triggers gp41-mediated fusion of the viral and cellular membranes.

A small number of monoclonal antibodies (mAbs) have been identified that neutralize a broad range of HIV-1 genetic subtypes. In addition, sera from some HIV-1-infected individuals display broadly neutralizing activity. Broadly neutralizing mAbs all bind the trimeric viral spike, and vaccines that mimic the native spike have the potential to elicit a broadly neutralizing response.

HIV-1 Env trimers are technically challenging to produce in purified form. Oligomeric gp140 polyproteins have been generated both by mutating the gp120–gp41 protease cleavage site and by expressing a wild-type polyprotein under conditions that do not foster cleavage. Trimeric forms of uncleaved gp140 have been additionally stabilized by the introduction of either heterologous trimerization domains or gp41–gp41 disulfide bonds. We previously described recombinant, proteolytically processed SOSIP gp140 proteins that incorporate three modifications to the native Env sequence: (1) truncation at the...
gp41 ectodomain, (2) incorporation of a gp120–gp41 intersubunit disulfide bond, and (3) a point mutation in gp41 to enhance trimer stability.\textsuperscript{15,16} The SOSIP gp140s also may contain a hexaarginine mutation at the gp120–gp41 cleavage site to promote efficient processing in transfected cell lines.\textsuperscript{17,18}

This approach has enabled the expression of proteolytically mature SOSIP gp140 proteins in a trimeric conformation.\textsuperscript{15,16,18,19} Initial studies examined SOSIP gp140 derived from HIV-1JR-FL, a prototypic subtype B primary isolate. In some rabbits, HIV-1JR-FL SOSIP gp140 elicited antibodies that neutralized HIV-1JR-FL and certain other primary HIV-1 isolates.\textsuperscript{18} In addition, we recently examined SOSIP gp140 proteins derived from a panel of contemporary subtype A HIV-1 isolates from sub-Saharan Africa.\textsuperscript{20} The SOSIP gp140 protein from one particular strain, KNH1144, formed unusually stable trimers.\textsuperscript{20}

In this report, we describe the isolation and characterization of substantially pure SOSIP gp140 trimers. The purification strategy exploited our prior observation that nonionic surfactants efficiently convert KNH1144 SOSIP gp140 aggregates to trimers.\textsuperscript{20} Here we report that Tween 20 rapidly converts KNH1144 SOSIP aggregates to trimers in a concentration-dependent, temperature-independent manner, and this surfactant effect was extended to SOSIP gp140 derived from HIV-15768, a subtype B isolate of vaccine interest.\textsuperscript{21} Surfactant-treated SOSIP gp140 had favorable antigenic characteristics, and electron microscopy studies revealed compact trimers that resemble virion-associated Env. Our studies provide a source of homogeneous, proteolytically mature HIV-1 envelope trimers for further structural and vaccine research.

**MATERIALS AND METHODS**

**Expression and purification of SOSIP gp140 and gp120**

The KNH1144 SOSIP gp140 construct with the hexaarginine (R6) mutation and the furin plasmid have been described.\textsuperscript{20} SOSIP gp140 and monomeric gp120 proteins were expressed using PPI4, which has been described previously.\textsuperscript{22} For a typical 8-liter preparation, human embryonic kidney (HEK) 293T cells were seeded into triple flasks (Corning Life Sciences, Acton, MA) at a density of 2.5 × 10\textsuperscript{7} cells/flask and cultured in Dulbecco’s modified Eagle medium (DMEM)/10% fetal bovine serum (FBS)/1% pen-strep (Invitrogen, Carlsbad, CA), with 1% L-glutamine 24 h prior to transfection. On the day of transfection, 270 μg KNH1144 SOSIP envelope DNA was mixed with 90 μg furin DNA (per flask) in Opti-MEM. Polyethyleneimine (PEI) (Polysciences Inc., Warrington, PA) was added stepwise (2 mg PEI:1 mg total DNA) and vortexed between each addition.\textsuperscript{23} The PEI/DNA solutions were incubated for 20 min at ambient temperature and then added to the flasks. Cells were incubated for 6 h at 32°C, 5% CO\textsubscript{2}, washed with warmed Dulbecco’s phosphate buffered saline (PBS) and then incubated in exchange media (DMEM/0.05% bovine serum albumin (BSA)/1% pen-strep) for 48 h at 32°C, 5% CO\textsubscript{2}. Supernatants were collected, and a protease inhibitor cocktail (Sigma, St. Louis, MO) was added according to the manufacturer’s instructions. Harvested supernatants were clarified using a 0.45-μm filter and concentrated 50-fold.

KNH1144 SOSIP gp140 trimers were purified by ammonium sulfate precipitation followed by affinity, size-exclusion, and ion-exchange chromatography. Concentrated cell culture supernatant was precipitated with an equal volume of 3.8 M ammonium sulfate. Ammonium sulfate was added with constant stirring, and samples then were immediately centrifuged at 4000 rpm, 4°C, for 45 min. The supernatant was diluted 4-fold with Dulbecco’s PBS without calcium, magnesium, or phenol red (PBS\textsuperscript{−}) at pH 7.25 and filtered using a 0.45-μm vacuum filter. The sample was then loaded onto a *Galanthus nivalis* agglutinin (GNA) lectin (Vector Laboratories, Burlingame, CA) column equilibrated with PBS\textsuperscript{−}. The column was washed with PBS\textsuperscript{−} until OD\textsubscript{280} reached baseline, followed by a second wash with 0.5 M NaCl PBS\textsuperscript{−} at pH 7.25 in order to remove BSA and other contaminant proteins. The column was then eluted with 1 M methyl-α-mannopyranoside (MMP, Sigma) in PBS\textsuperscript{−} starting with flowing one-half column volume (CV) and passing the purification for 1 h. Flow was then restarted, and eluate fractions were pooled and concentrated using a Vivaspin 100,000 Da molecular weight cutoff (MWCO) concentrator (Vivascience, Edgewood, NY) spun at 1000 × g. The concentrate was applied over a Superdex 200 size-exclusion chromatography (SEC) column (GE Healthcare, Piscataway, NJ) equilibrated in 20 mM Tris, pH 8.2, 200 mM NaCl (TN-200), and resolved at 0.4 ml/min. Trimer-containing fractions were pooled and diluted to 75 mM NaCl with 20 mM Tris, pH 8. The diluted SEC pool was then applied over a 1 ml HiTrap diethyl aminoethyl (DEAE) FF column (GE Healthcare), equilibrated in 20 mM Tris, pH 8, 75 mM NaCl (TN-75). The column was washed with TN-75 until the OD\textsubscript{280} reached baseline and then eluted with 20 mM Tris, 300 mM NaCl, pH 8. To maximize trimer yield, the DEAE flow-through was reapplied over the column (equilibrated in TN-75), and typically 20–30% more trimer was recovered in this manner. Trimer-containing fractions were pooled and the concentration was determined by densitometry on a reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) gel using JR-FL gp120 as a standard. KNH1144 gp120 was expressed and purified using GNA lectin, Q Sepharose (GE Healthcare), and Superdex 200 size-exclusion chromatographies essentially as described.\textsuperscript{19}

HIV-15768.a is a CCR5-tropic, subtype B strain isolated from an acutely infected patient within 1 month of seroconversion.\textsuperscript{21} A construct expressing 5768 SOSIP gp140 containing the R6 mutation was generated as described previously.\textsuperscript{16,20} 5768 SOSIP gp140 was purified as described above for KNH1144 SOSIP with the following exceptions: the 293T culture supernatants were concentrated 63-fold prior to precipitation with ammonium sulfate, and the SEC run buffer was 20 mM histidine, 150 mM, pH 6.0. DEAE was not used to purify 5768 SOSIP gp140.

**SDS–PAGE and BN–PAGE**

SDS–PAGE analyses were performed using 4–12% Bis-Tris NuPage gels (Invitrogen). Blue native PAGE (BN–PAGE) was performed as described.\textsuperscript{19} Thyroglobulin (670 kDa) and ferritin (440 kDa) were used as molecular weight standards. Coomassie staining was performed using either the SimplyBlue SafeStain or Easy-to-Use Coomassie G-250 Stain (Invitrogen). Silver staining was performed with the SilverQuest kit (Invitrogen).
Western blot analysis was performed with mAb AR3119 antibody (CA13, Centralised Facility for AIDS Reagents, Herts, United Kingdom).

Effect of Tween 20 on Env aggregates

Aliquots (1 μg) of purified SOSIP gp140 were treated with Tween 20 and then analyzed by BN–PAGE and Coomassie staining. In a first study, KNH1144 SOSIP gp140 was treated with Tween 20 at concentrations ranging from 0 to 0.1% (v/v) for 1 h at ambient temperature (~23°C) prior to analysis. Alternatively, samples were incubated with Tween 20 at a final concentration of 0.05% (v/v) for 5 min and 10 min at ambient temperature prior to analysis. Temperature effects were studied by incubating samples in 0.05% (v/v) Tween 20 at 0°C (on ice), ambient temperature, or 37°C for 10 min. 5768 SOSIP gp140 was treated with 0.05% Tween 20 for 10 min at ambient temperature.

Similarly, 1 μg of purified gp120 monomer or 0.5 μg purified α2-macroglobulin (A2M, Sigma, St. Louis, MO) was either untreated or incubated with Tween 20 at a final concentration of 0.05% for 10 min at ambient temperature and then analyzed by BN–PAGE. A2M is a homotetrameric glycoprotein comprised of ~185 kDa subunits.

Analytical size-exclusion chromatography

Analytical runs were performed at 4°C on an AKTA fast protein liquid chromatography (FPLC) system (GE Healthcare). Each run was performed at least twice. A Superdex 200 10/300 GL column was equilibrated in 20 mM Tris, pH 8, 0.5 M NaCl (TN-500) and calibrated with the following molecular weight standard proteins (GE Healthcare): thyroglobulin 669,000 Da, ferritin 440,000 Da, BSA 67,000 Da, and RNase A 13,700 Da. A standard curve was generated by plotting the observed retention volumes of the standard proteins against the log values of their predicted molecular weights.

Purified KNH1144 SOSIP gp140 (8–10 μg) was treated with Tween 20 at a final concentration of 0.05% for 10–30 min at ambient temperature. Samples were then applied over the Superdex 200 column equilibrated with TN-500 containing 0.05% Tween 20 (TN-500) and resolved at 0.4 ml/min, collecting 0.4 ml fractions. Trimer-containing fractions were then analyzed by BN–PAGE, followed by silver stain. Fractions were also separated by BN–PAGE, followed by Western blot analysis with ARP 3119 antibody. In addition, 10–14 μg of purified KNH1144 gp120 or JR-FL gp140 was applied over the Superdex 200 column equilibrated in TN-500 and resolved at a flow rate of 0.4 ml/min.

 Lectin ELISA and immunoprecipitation

Human mAbs b6, b12,25 and 2G12,26 and HIVIg27 were obtained from Dr. Dennis Burton (The Scripps Research Institute, La Jolla, CA) or Dr. Herman Katinger (University of Natural Resources and Applied Life Sciences, Vienna, Austria). For the lectin-capture ELISA, anti-Env antibodies 2G12, b6, b12, and HIVIg were used. The CD4–IgG2 fusion protein was prepared as previously described.28 ELISA plates were coated overnight at 4°C with Lens culinaris (lentil) lectin (Sigma) at 10 μg/ml concentration, and the assay was performed at ambient temperature thereafter. Plates were washed with PBS twice and blocked with SuperBlock (Pierce). Excess blocking agent was washed off with PBS. Samples were added at 0.3 μg/ml to the plates for four h. The plates were washed 4 times with PBS and incubated with anti-Env antibodies or CD4–IgG2 starting at 10 μg/ml in PBS/5% milk. Then 4× serial dilutions were performed and incubations were performed for 3 h. Plates were washed six times and goat antihuman IgG (H + L) alkaline phosphatase conjugate secondary antibody (Jackson Immuno-Research) was added at 1/4000 dilution in PBS/5% milk. Plates were washed four times and developed using the AMPAK detection system (Dako Cytomation, Carpinteria, CA) as per the manufacturer’s instructions. Immunoprecipitation studies were performed as previously described20 with the above mAbs and with mAbs to the CD4-binding site (15e,30 48d,30 and X531) and to CD4-induced epitopes (17b,30 48d,30 and X531).

DEAE anion-exchange chromatography

Purified KNH1144 SOSIP gp140 trimers, treated either with or without 0.05% Tween 20, in TN-75 buffer were applied to a 1-ml DEAE HiTrap FF column (equilibrated in TN-75) at 0.25 ml/min at ambient temperature, washed with TN-75 at 0.5 ml/min, and eluted with TN-300. Equal amounts of flowthrough, wash, and elution fractions were analyzed via BN–PAGE, followed by Coomassie G-250 stain.

Electron microscopy

Negative-stain electron microscopy was performed as previously described.32,33 Because this technique is incompatible with Tween 20, 20 μl of purified KNH1144 SOSIP gp140 (0.5 mg/ml in TN-300 with 0.05% Tween 20) was dialyzed against BSB (0.1 M H2BO3, 0.025 M Na2B4O7, 0.075 M NaCl, pH 8.3) and subsequently depleted of Tween 20 using the Mini Detergent-OUT kit (Calbiochem, La Jolla, CA), as described by the manufacturer. Two micrograms of Tween 20-depleted protein were diluted in 200 μl BSB, and affixed to a carbon support membrane. The membrane was stained with 1% uranyl formate, and mounted on 600 mesh copper grids for analysis. Electron micrographs were recorded at 100,000× magnification at 100 kV on a JOEL JEM 1200 electron microscope. Trimmers were measured and analyzed statistically using Image-Pro Plus software (www.mediacy.com).

RESULTS

Expression and purification of SOSIP gp140 trimers

Our purification scheme for KNH1144 SOSIP gp140 involved ammonium sulfate precipitation followed by three chromatography steps: GNA lectin affinity, Superdex 200 size exclusion, and DEAE anion exchange. While the GNA lectin column efficiently and selectively captured KNH1144 SOSIP gp140, significant dissociation of trimers into dimers and monomers occurred during elution with MMP (data not shown). Monomers and dimers were effectively removed using SEC and DEAE chromatography, respectively.

Purified KNH1144 SOSIP gp140 migrated as a single 140-kDa gp140 band on a nonreducing SDS–PAGE gel (Fig. 1A).
The absence of a significant gp120 band under nonreducing conditions indicates that the intersubunit disulfide bond is intact. No disulfide-linked SOSIP gp140 aggregates were detected by either Coomassie blue staining or Western blotting under nonreducing conditions. When reduced, KNH1144 SOSIP gp140 yielded a 120-kDa gp120 band in SDS–PAGE (Fig. 1A). The absence of a gp140 band under reducing conditions indicates that the purified protein is fully cleaved at the gp120–gp41 proteolytic site.

Purified KNH1144 gp120 migrated as a single band of approximately 120 kDa in SDS–PAGE (Fig. 1A). Common serum contaminants, such as A2M and BSA, collectively comprised <5% of the purified KNH1144 gp120 and SOSIP gp140 proteins.

When analyzed by BN–PAGE, purified KNH1144 SOSIP gp140 migrated predominantly as a trimer, consistent with our prior results for partially purified protein. Dimeric and monomeric forms of gp140 were not evident. However, an additional >670-kDa band was observed in the absence of Tween 20 (Fig. 1B), and this band was determined by Western blotting to be high-molecular-weight (HMW) aggregates of SOSIP gp140 (data not shown). The relative abundance of HMW aggregates ranged from 10% to 40% among the different lots of purified product.

**Surfactant treatment**

Treatment of purified KNH1144 SOSIP gp140 with mild surfactant (0.05% Tween 20) quantitatively converted the HMW aggregates into trimers (Fig. 1B). There was no appreciable dissociation of trimers into monomers or dimers, consistent with our preliminary observations. Despite its nonionic nature, Tween 20 modestly but measurably increased the mobility of the trimers in BN–PAGE (Fig. 1B). One possible explanation for the faster migration of Tween-treated SOSIP trimers is that the surfactant causes SOSIP to adopt a more compact conformation. As a second possible explanation, Coomassie blue may bind to Tween 20 molecules that are bound to SOSIP, giving SOSIP a greater overall net charge within the BN–PAGE gel. These possibilities are not mutually exclusive. Surfactant treatment also increased the staining intensity of SOSIP gp140. Tween 20 had no obvious effect on the mobility of gp120 in BN–PAGE (Fig. 1B) but modestly increased its staining intensity in some cases (data not shown).

Since surfactant treatment provided a facile means to obtain homogeneous trimers, we further characterized this process. A preparation containing ~30% HMW aggregates was treated with Tween 20 at concentrations ranging from 0.0001% to 0.1% (v/v). Concentrations of 0.01% to 0.1% dissociated aggregates into trimers (Fig. 2A). Trace amounts of aggregates were present at 0.01% Tween 20, and no conversion was observed at 0.001% or 0.0001% (Fig. 2A). Therefore, 0.01% represents a threshold concentration for conversion. The kinetics of dissociation were studied by incubating SOSIP gp140 with 0.05% Tween 20 for 5 and 10 min at ambient temperature, prior to analysis by BN–PAGE. As shown in Fig. 2B, aggregates were completely eliminated at both 5 min and 10 min. We next examined the effect of temperature on the dissociation of aggregates. Efficient conversion of aggregates to trimers was observed within 10 min at temperatures of 0°C, 23°C, and 37°C (Fig. 2C). Finally, similar results were obtained when Tween 80 was used in place of Tween 20 (data not shown).

Surfactant treatment was also applied to purified SOSIP gp140 derived from HIV-15768, a subtype B isolate. 5768 SOSIP gp140 trimers show intermediate stability relative to the more stable KNH1144 and less stable JR-FL SOSIP gp140 proteins. Both dimeric and monomeric gp140 bands are clearly visible in a preparation of purified 5768 SOSIP gp140 (Fig. 2D).
Nevertheless, in the absence of surfactant, the main contaminant is HMW aggregates, and these aggregates were reduced to trace levels by 0.05% Tween 20 (Fig. 2D). As was observed for KNH1144 SOSIP gp140, surfactant increased the mobility of 5768 SOSIP gp140 trimers. 5768 gp140 monomers and dimers also showed increased mobility. Importantly, there was no apparent increase in the amounts of the monomeric and dimeric gp140 bands, suggesting that surfactant efficiently converted aggregates to trimers for this subtype B SOSIP gp140 protein.

To test if surfactant dissociated an unrelated multisubunit protein, 0.05% Tween 20 was added to A2M, which is a non-covalent tetramer of identical 185-kDa subunits. Treatment did not affect the oligomeric state of A2M, although there was a small increase in staining intensity of the protein (Fig. 3A). We next treated a preparation of KNH1144 SOSIP gp140 containing >80% HMW aggregates. Tween 20 was effective in converting the aggregates to trimers (Fig. 3B). Finally, a preparation composed of a mixture of KNH1144 SOSIP HMW aggregates, dimers, and monomers was treated with Tween 20. In this case, Tween 20 converted HMW aggregates to trimers but had no obvious effect on the amount of monomers or dimers (Fig. 3B). The findings indicate that Tween 20 selectively dissociates HMW aggregates into trimers.

**SEC analysis**

Superdex 200 SEC was performed as a second means to characterize KNH1144 gp120 and SOSIP gp140. In addition, monomeric JR-FL gp120 was also analyzed as a control. KNH1144 gp120 and JR-FL gp120 each eluted at an apparent molecular weight of 210 kDa (data not shown), consistent with our prior findings for JR-FL gp120.19 The high apparent molecular weight in SEC is thought to reflect the extended nature of the glycan chains on Env.

In initial studies with surfactant-treated KNH1144 SOSIP gp140, we observed that HMW aggregates reformed when Tween 20 was not included in the SEC run buffer. Therefore, SEC was performed in the presence of 0.05% Tween 20, and the column fractions were analyzed by BN–PAGE and silver staining. As shown in Fig. 4, trimers comprised the major SEC elution peak. The average retention volume (9.4 ml) corre-
sponds to an apparent molecular weight of ~520 kDa, similar to that reported previously for JR-FL SOSIP gp140 trimers.\textsuperscript{16} The SEC and BN–PAGE data concur that surfactant-treated KNH1144 SOSIP gp140 is trimeric.

**Effect of surfactant on antigenicity**

Previously, we reported that unpurified KNH1144 SOSIP gp140 was immunoprecipitated by the neutralizing agents 2G12, b12, and CD4–IgG2, and by the nonneutralizing mAb b6.\textsuperscript{20} In preliminary experiments, we immunoprecipitated purified trimers with 2G12, IgG1b12, CD4–IgG2, b6, D20, 15e, 17b, X5, and 48d. Consistent with our observations on unpurified material, purified KNH1144 SOSIP trimers were efficiently immunoprecipitated with 2G12, IgG1b12, CD4–IgG2, and b6. In addition, both trimers and gp120 monomers were immunoprecipitated with mAbs 17b and X5 in the presence but not the absence of soluble CD4, but neither trimers nor monomers were immunoprecipitated with mAb 48d under any circumstance. The CD4 binding site mAbs 15e and D20 did not immunoprecipitate either KNH1144 SOSIP gp140 trimers or gp120 monomers (data not shown).
Effects of Tween 20 on the ionic properties of KNH1144 SOSIP gp140 and A2M

We used DEAE anion-exchange chromatography to examine the effect of Tween 20 on the ionic properties of SOSIP gp140 and A2M. For this, KNH1144 SOSIP gp140 was spiked with A2M, and the mixture either was treated with Tween 20 or was left untreated prior to DEAE chromatography. Column fractions were analyzed via BN–PAGE. As expected, untreated SOSIP gp140 trimers bound to the DEAE column and were recovered in the eluate (Fig. 6A). In the presence of surfactant, however, KNH1144 SOSIP gp140 flowed through the column (Fig. 6B). In contrast, A2M bound DEAE in the presence or absence of Tween 20 (Fig. 6). In similar experiments, Tween 20 did not affect binding of BSA to DEAE (data not shown). Therefore, Tween 20 selectively affects both the oligomeric state and the charge properties of KNH1144 SOSIP gp140.

Electron microscopy of KNH1144 SOSIP gp140

We next studied the appearance of purified SOSIP gp140 using negative-stain electron microscopy. As shown in Fig. 7, KNH1144 SOSIP gp140 displayed a regular compact morphology with approximate 3-fold symmetry. As is typical, different areas on the grid display different staining characteristics including areas of shallow and deep stain. To estimate the size of the trimers, 78 trimers from the deep-stain regions (Fig. 7) were scored, yielding a diameter estimate of 11.6 nm ± 1.8 nm. In addition, 70 spikes in the shallow-stain regions (data not shown) were also scored; the resulting diameter estimate was 13.5 ± 1.7 nm. The shallow stain slightly overestimates protein size, whereas the deep stain slightly underestimates it. Therefore, the true diameter is likely to be 12–13 nm, in line with that observed for virion-associated simian immunodeficiency virus (SIV) Env spikes as measured using both similar negative-staining electron microscopy procedure and cryoelectron microscopy methods.34,35 The electron microscopy, SEC, and BN–PAGE data are consistent in demonstrating that purified KNH1144 SOSIP gp140 is a trimer.

DISCUSSION

We describe the isolation and initial characterization of proteolytically mature HIV-1 envelope trimers. To our knowledge, KNH1144 SOSIP gp140 represents the first HIV-1 envelope to be produced and isolated as essentially homogeneous cleaved trimers. Electron microscopy and antigenicity studies demonstrate that the purified protein forms compact trimers that reproduce a number of features of the native envelope spike. Isolation of homogeneous trimer was enabled by the finding that mild treatment with nonionic surfactant quantitatively converted HMW aggregates to trimers, and the surfactant effect was generalizable to another subtype B HIV-1 Env. The purified, cleaved Env trimers are being utilized for further structural analyses and for vaccine research.

Compared to other subtype A and subtype B SOSIP gp140 proteins,16,18–20 KNH1144 SOSIP trimered more efficiently, and the trimers proved stable to purification. The stability of the KNH1144 Env was identified empirically; however, its sequences may prove useful in understanding the determinants of...
Env trimerization, at least in the context of soluble gp140 proteins. Initial studies have demonstrated that trimer stability is influenced by residues in the N-terminal heptad-repeat region of gp41, and the stability of JR-FL gp140 trimers was improved by substitution with five residues from this region of KNH1144.36

In the absence of surfactant, purified KNH1144 SOSIP gp140 contained 10–40% HMW aggregates in addition to trimers. Fortuitously, the HMW SOSIP aggregates were non-covalently associated and were rapidly and quantitatively converted to trimers in the presence of ≥0.01% Tween 20. In contrast, JR-FL SOSIP gp14016 and other recombinant HIV-1 gp140 proteins10,13,14,37 form HMW aggregates that include disulfide-linked species, which reflect aberrant linkages between the ~20 cysteines within gp120 and gp41. Such aggregates are dissociated only under reducing and denaturing conditions, and can be challenging to separate from the trimers. In addition, Tween 20 dissociated the HMW aggregates present in purified SOSIP gp140 derived from the subtype B isolate HIV-15766. Therefore, surfactant treatment may have utility in iso-

FIG. 5. Effect of Tween 20 on antigenicity. Lectin ELISA of untreated and Tween 20-treated KNH1144 SOSIP gp140. Untreated or Tween 20-treated proteins were bound to lectin-coated ELISA plates and probed with 2G12, b6, b12, CD4-IgG2, and HIVIg. The y-axis represents the colorimetric signal at OD492 and the x-axis represents antibody concentration in µg/ml. (A) Fraction containing >80% HMW aggregates pretreatment. (B) Fraction containing 10–15% HMW aggregates pretreatment. HIVIg was not tested with this fraction due to a limited availability of material.
lating highly purified forms of SOSIP gp140 trimers derived from different strains and genetic subtypes of HIV-1.

A concentration of 0.01% Tween 20 represented a threshold for efficient conversion of SOSIP HMW aggregates to trimers. This and higher amounts of Tween 20 had no effect on oligomerization of α2-macroglobulin, as expected given the mild nature of this surfactant. Indeed, Tween 20 and Tween 80 commonly are used to stabilize protein formulations in order to reduce the formation of aggregates.43–42 However, these surfactants are much less effective in dissociating preexisting protein aggregates. This observation highlights both the novelty of our finding and the unusually labile nature of the SOSIP HMW aggregates.

The purification scheme for KNH1144 SOSIP gp140 incorporates a number of improvements over the process previously described for JR-FL SOSIP gp140. First, the lectin column was optimized by adding a static incubation in elution buffer. This incubation reduced trimer dissociation and improved trimer yield. Galanthus nivalis lectin binds α1–3 and α1–6 mannose linkages, which are internal and not terminal linkages.43 We reasoned that SOSIP trimers are likely to bind multivalently to the lectin column, and the effective off-rate would be slower

FIG. 6. Effect of Tween 20 on KNH1144 SOSIP gp140 binding to DEAE. Purified KNH1144 SOSIP gp140 trimer was spiked with α2-macroglobulin (A2M) and then either left untreated or treated with Tween 20. Samples were then applied over a DEAE HiTrap FF 1 ml column. Load (Lane 2), flow-through (Lanes 3 and 4), wash (Lane 5), and eluate (Lane 6) fractions were collected and analyzed by BN–PAGE, followed by Coomassie G-250 stain. Lane 1 contains molecular weight markers. (A) Untreated sample. (B) Sample treated with 0.05% Tween 20.
than those for smaller or monovalent ligands. The extended incubation may permit a greater number of trimers to fully dissociate from the column in the absence of shear forces and concentration gradients that would be generated by buffer flow.

Second, an anion-exchange column was introduced. DEAE chromatography effectively resolved SOSIP trimers from dimers that were not fully removed by SEC. Surprisingly, retention of SOSIP trimers on DEAE was profoundly altered by Tween 20. Trimers bound DEAE in the absence but not the presence of 0.05% Tween 20, whereas retention of contaminants was unaffected by Tween 20. The unique behavior of SOSIP gp140 over DEAE can be exploited for purification purposes. As a nonionic surfactant, Tween 20 is likely to exert an indirect effect on the charge properties of KNH1144 SOSIP gp140, and this issue is being addressed in ongoing studies.

Purified KNH1144 SOSIP gp140 demonstrated favorable antigenic properties, consistent with our previous findings for unpurified material. Tween 20 had no obvious effect on the

FIG. 7. Negative stain electron micrograph gallery of KNH1144 SOSIP gp140. Bar = 50 nm.
antigenicity of a preparation containing predominantly pure trimers (<15% HMW aggregates pretreatment). However, antigenicity is an imperfect predictor of immunogenicity, and a study in rabbits has been initiated to compare the immunogenicity of KNH1144 SOSIP gp140 in the presence and absence of Tween 20.

Treatment of a preparation containing >80% HMW aggregates increased exposure of the CD4-binding site, suggesting that aggregates form in a manner that occludes this region of Env. Other epitopes were unaffected, indicating that dissociation of aggregates does not entail a global change in Env conformation. Collectively, the BN–PAGE, SDS–PAGE, and antigenicity data are consistent with a view that the HMW aggregates represent two or more native trimers that are associated via weak hydrophobic and/or other noncovalent interactions.

A distinguishing feature of KNH1144 SOSIP gp140 is its compact nature. Other recombinant HIV-1 gp140 proteins appear in electron micrographs as either elongated, loosely associated subunits. In comparison, KNH1144 SOSIP gp140 more closely resembles native Env in its dimensions and configuration. We are performing immunoelectron microscopy analyses of this protein in complex with a panel of mAbs to delineate the spatial relationships between known neutralization epitopes on the trimers. We will also investigate, in comparative studies, whether cleaved and uncleaved forms of KNH1144 gp140 differ in their appearance in electron microscopy.

KNH1144 SOSIP gp140 and the methods described herein provide the first reliable source of homogeneous, proteolytically mature HIV-1 envelope trimers. The trimers possessed favorable antigenic and biophysical properties, and this protein may have value in further elucidating structure–function relationships within the HIV-1 envelope. In addition, based on its propensity to form stable trimers, KNH1144 SOSIP may serve as a template for additional studies into HIV-1 envelope structure and biology.

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