Abstract: This invention provides processes of purifying and recovering trimers of a retroviral envelope (Env) protein from a preparation comprising trimers of the retroviral Env protein and high molecular weight aggregates thereof. Non-ionic detergent is used in the processes to obtain a purified trimer product, which is separated from aggregates. The process further comprises treating the preparation with a non-ionic detergent so as to thereby convert the high molecular weight aggregates into trimers, and purifying and recovering the trimers from the preparation so treated.
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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.
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Throughout this application, certain publications are referenced. Full citations for these publications may be found immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention relates.

**BACKGROUND OF THE INVENTION**

According to the 2005 World Health Organization AIDS epidemic update, there are over 40 million people infected with human immunodeficiency virus (HIV) worldwide, with close to 5 million newly infected cases reported just last year (1). Among one of the hardest hit areas is sub-Saharan Africa, with over 25 million people living with HIV and about 10% dying of AIDS-related illnesses. It has been widely recognized and accepted that prophylactic measures in the form of an HIV vaccine, in addition to therapeutic medicines, need to be implemented to curtail the spread of AIDS globally. An effective HIV vaccine should demonstrate an ability to elicit neutralizing antibodies (nAb) that are capable of blocking the fusogenic interaction and entry of HIV with the CD4 receptor on CD4+ helper T cells, mediated by the cell surface viral Env glycoproteins, gp120 and gp41. Since the genetic polymorphism of the HTV-I gag and env genes are diverse and constantly evolving due to rapid mutation within individuals (2), the nAbs targeting the gp120 and gp41 envelope proteins on the viral surface should be capable of blocking the viral interaction with the CD4 receptor and should neutralize viruses from a broad range of subtypes without discrimination.

One logical design of recombinant Env vaccine candidates is to base the vaccine sequence on currently existing HIV-I isolates prevalent in the infected population. To this end, several oligomeric env proteins from several different subtypes or "clades" have been described, with subtype B sequences serving as a basis for the majority of those that have been reported (3-11,
The oligomeric Env protein complex on the surface of the virus is comprised of a gpl20-gp41 heterodimer present in a homotrimer configuration (held together via non-covalent interactions), resembling a "spike" structure. These glycoproteins are derived from a gpl 60 precursor protein, which undergoes processing and cleavage in the cell to generate gpl 20 and gp41 heterodimers that are then targeted to the surface of the HIV viral envelope (12, 13). Fusion of the virus with the CD4+ cell membrane and oligomerization of the trimer spike is mediated by the gp41 glycoprotein, which is tethered to the virion surface via its transmembrane domain (12, 13).

It has been reasoned that design of a recombinant vaccine should mimic the native trimer spike of the HIV envelope against which nAbs are generated. Since the native Env trimer is technically challenging to produce in a recombinant form, modified versions of the trimer that could serve as potential vaccine templates have been reported. One typical modification is truncation of the gp41 transmembrane domain from the precursor gpl 60 to yield gpl 40 proteins in a soluble form. However, following processing and cleavage, the resulting gpl20 and gp41 ectodomain or gp41 Ec to (lacking the transmembrane domain) have been shown to form unstable associations and tend to dissociate into their respective monomeric subunits (13, 14).

To address these issues, subtype B HIVJR-FL Env was used as a template into which a disulfide bond was introduced between gpl20-gp4IEc to subunits (SOS gpl40), followed by a further modification to gpl Ec to (I559P mutation), which successfully allowed for the expression of stable, cleaved and fully processed oligomeric gpl 40 proteins in a trimeric conformation (SOSIP gpl40) (8-11, 15-17). While immunization of rabbits performed with the engineered HIV-1 JR-FL SOSIP gpl40 elicited antibodies capable of neutralization, the activity was limited primarily to the homologous strain, with only a modest and limited ability to neutralize across different HIV-1 primary isolates (11).

While the SOSIP technology addresses stability and expression, another issue that has limited production and purification of the recombinant trimers has been the spontaneous association of the oligomeric gpl40 proteins into aberrant "aggregate" species (3, 9, 11, 18). These aggregate species, typically identified by their reduced mobility on blue native PAGE (BN-PAGE) and non-reduced SDS-PAGE have been difficult to purify from the SOSIP gpl 40 trimer without compromising yield and/or stability of the trimer. Attempts to fully characterize the aggregate have been limited and their true nature remains elusive.
To explore a wider variety of oligomeric Env proteins that elicit higher breadths of cross-neutralization activity and serve as potential vaccine immunogens, a panel of subtype A sequences from HIV-I primary isolates in sub-Saharan Africa was described (19). The Env proteins from these sequences were expressed as SOSIP gpl40 proteins, with a further engineered mutation at the gpl20-gp41_eC cleavage site (R6) for enhanced furin cleavage (>95% efficiency) to yield soluble, stable and fully processed gpl40 trimers.

To address a need in the art for HIV Env proteins that may serve as stable immunogens and may mimic native HIV Env protein for the production of broadly reactive neutralizing antibodies, the present invention provides novel modified HIV-I Env protein isolates which are purified as complex trimeric forms that closely resemble the structural Env spikes of the native HIV envelope. A novel recovery process for obtaining trimeric Env gpl40 protein is also provided.

**SUMMARY OF THE INVENTION**

Described herein is the purification and biochemical characterization of HIV-I Env isolates, e.g. a modified subtype A KNHI 144 SOSIP R6 gpl40, derived from a contemporary East African subtype A HIV-I primary isolate, and a modified subtype B 5768.4 SOSIP R6 gpl40, using novel methodologies that are different from currently implemented purification procedures. The purified KNHI 144 SOSIP R6 gpl40 and 5768.4 SOSIP R6 gpl40 are trimers, as assessed by BN-PAGE and size exclusion chromatography (SEC). In addition, described herein are newly discovered effects of non-ionic detergents, such as Tween® 20, on aggregates of the KNHI 144 SOSIP R6 and the 5768.4 SOSIP R6 gpl40 proteins, thus providing insights into the nature of the aggregate species. The effects of Tween® 20 treatment on the antigenic properties of KNHI 144 SOSIP R6 gpl40 aggregates and trimers are described in accordance with the invention. New processes of purifying trimers (greater than 90% trimers) from high molecular weight aggregates of such trimers are provided in accordance with this invention. Finally, digital imaging based on negative stain electron microscopy reveals the structure of purified KNHI 144 SOSIP R6 gpl40 as trimeric oligomers in the virtual absence of monomeric and dimeric forms.

This invention provides a process of recovering trimers of a retroviral envelope (Env) protein from a preparation comprising trimers of the retroviral Env protein and high molecular weight aggregates thereof, which comprises treating the preparation with a non-ionic detergent so as to thereby convert the high molecular weight aggregates into trimers, and recovering the trimers from the preparation so treated.
This invention also provides a process of recovering trimers of a retroviral envelope (Env) protein from a preparation comprising trimers of the Env protein and high molecular weight aggregates thereof which comprises: (a) subjecting the preparation to lectin chromatography to obtain a trimer-enriched eluate in which aggregate content is reduced; (b) applying the eluate of step (a) to a first diethylaminoethyl (DEAE) sepharose chromatography column in the absence of non-ionic detergent to obtain elution fractions further enriched in trimers; (c) applying trimer-enriched elution fractions from the first DEAE sepharose chromatography column of step (b) to a second DEAE sepharose chromatography column in the presence of non-ionic detergent; and (d) collecting the flow through and wash fractions from the second DEAE sepharose chromatography column in the presence of non-ionic detergent, so as to thereby recover the trimers of the retroviral Env protein.

This invention further provides a process of recovering trimers of a retroviral envelope (Env) protein from a preparation comprising trimers of the retroviral Env protein and high molecular weight aggregates thereof, which comprises: (a) subjecting the preparation to lectin affinity chromatography to obtain an eluate enriched in trimers and essentially separated from monomer and dimer aggregates and macroglobulin; and (b) subjecting the eluate of step (a) to a first diethylaminoethyl sepharose chromatography column in the absence of non-ionic detergent so as to further remove aggregates in the flow through; and (c) subjecting the eluate of step (b) to a second diethylaminoethyl sepharose chromatography column in the presence of non-ionic detergent so as to recover the trimers of the retroviral Env protein.

This invention also provides a process of recovering trimers of a retroviral envelope (Env) protein from a preparation comprising trimers of the Env protein and high molecular weight aggregates thereof which process comprises subjecting the preparation to size exclusion chromatography in the presence of a non-ionic detergent so as to convert the high molecular weight aggregates into trimers, and recovering the trimers from the preparation subjected to the chromatography.

This invention further provides a process of recovering trimers of a retroviral envelope (Env) protein from a preparation comprising trimers of the Env protein and high molecular weight aggregates thereof which comprises (a) subjecting the preparation to size exclusion chromatography to obtain eluted fractions; (b) subjecting the eluted fractions of step (a) to anion exchange chromatography; and (c) treating the fractions of step (b) with a non-ionic detergent, so as to thereby recover the trimers of the retroviral Env protein.
This invention also provides a process of recovering trimers of a retroviral envelope (Env) protein from a preparation comprising trimers of the retroviral Env protein and high molecular weight aggregates thereof which comprises (a) subjecting the preparation to lectin affinity chromatography to obtain eluted fractions; and (b) subjecting the fractions of step (a) to size exclusion chromatography in the presence of a non-ionic detergent, so as to thereby recover the trimers of the Env protein.

This invention further provides a process of recovering trimers of a retroviral envelope (Env) protein from a preparation comprising trimers of the Env protein and high molecular weight aggregates thereof which comprises (a) subjecting the preparation to lectin affinity chromatography to obtain fractions comprising monomers, dimers, and trimers of the Env protein; (b) subjecting the fractions of step (a) to size exclusion chromatography to obtain fractions comprising dimers and trimers of the Env protein; (c) subjecting the fractions of step (b) to anion exchange chromatography to obtain a fractions in which trimers of the Env protein are separated from dimers; and (d) treating the fractions of step (c) with non-ionic detergent, so as to thereby recover the trimers of the Env protein.

This invention also provides a process wherein the Env protein comprises (a) a first polypeptide which comprises consecutive amino acids, the sequence of which corresponds to the sequence of a modified gp120 envelope polypeptide portion of a gp41 envelope of an HTV-I KNHI 144 isolate, or a quasi-species thereof; and (b) a second polypeptide which comprises consecutive amino acids, the sequence of which corresponds to the sequence of a modified gp41 ectodomain polypeptide portion of the gp41 envelope of the HIV-I KNHI 144 isolate or such quasi-species thereof, the sequence of said modified gp120 envelope polypeptide portion and said modified gp41 ectodomain polypeptide portion of said HIV-I KNHI 144 isolate being as set forth in SEQ ID NO.2 and SEQ ID NO:3, respectively, said modified gp120 envelope polypeptide portion comprising a cysteine at amino acid position 511 and said modified gp41 ectodomain polypeptide portion comprising a cysteine at amino acid position 617 and a proline at amino acid position 571, wherein (i) the amino acid positions are numbered by reference to SEQ ID NO:1, (ii) the modified gp120 envelope polypeptide portion further comprises a mutated furin recognition sequence, and (iii) the modified gp120 polypeptide portion and the modified gp41 ectodomain polypeptide portion are bound to one another by a disulfide bond between the cysteine at amino acid position 511 and the cysteine at amino acid position 617.

This invention further provides a process wherein the Env protein comprises (a) a first polypeptide which comprises consecutive amino acids, the sequence of which corresponds to the
sequence of a modified gpl20 envelope polypeptide portion of a gpl40 envelope of an HIV-I 5768.4 isolate, or a quasi-species thereof; and (b) a second polypeptide which comprises consecutive amino acids, the sequence of which corresponds to the sequence of a modified gp41 ectodomain polypeptide portion of the gpl40 envelope of the HIV-I 5768.4 isolate or such quasi-species thereof, the sequence of said modified gpl20 envelope polypeptide portion and said modified gp41 ectodomain polypeptide portion of said HIV-I 5768.4 isolate being as set forth in SEQ ID NO:5 and SEQ ID NO:6, respectively, said modified gpl20 envelope polypeptide portion comprising a cysteine at amino acid position 519 and said modified gp41 ectodomain polypeptide portion comprising a cysteine at amino acid position 625 and a proline at amino acid position 579, wherein (i) the amino acid positions are numbered by reference to SEQ ID NO:4, (ii) the modified gpl20 envelope polypeptide portion further comprises a mutated furin recognition sequence, and (iii) the modified gpl20 polypeptide portion and the modified gp41 ectodomain polypeptide portion are bound to one another by a disulfide bond between the cysteine at amino acid position 519 and the cysteine at amino acid position 625.

**BRIEF DESCRIPTIONS OF THE FIGURES**

**FIGURE 1**: Analysis of purified KNH1 144 SOSIP R6 gpl40 trimer and gpl20 monomer. Purified KNH1 144 gpl20 monomer (left panel, gp!20) and SOSIP R6 gpl40 trimer were analyzed by reducing (left panel, **SOSIP R6, Red**) and non-reducing SDS-PAGE (left panel, **SOSIP R6, NK**). Proteins were visualized by Coomassie G-250 stain. Purified trimer was also analyzed via ARP3 119 western blot on non-reducing SDS-PAGE to examine presence of SDS-insoluble aggregates (**middle** panel, *Anti-Env blot*). The numbers on the left represent the migratory positions of the molecular weight standard proteins. The **right** panel shows BN-PAGE analysis of purified trimer, either untreated or treated with Tween® 20 (**SOSIP R6, +/- lanes**) and purified gpl20 monomer in absence or presence of Tween® 20 treatment (**gpl20, +/- lanes**). Arrows indicate high molecular weight (HMW) aggregate, trimer and gpl20 monomer species. *N* stands for the 669k thyroglobulin and 440k ferritin molecular weight protein standards.

**FIGURES 2A-2D**: Tween® 20 conversion experiments. (A) Dose response: Purified KNH1 144 SOSIP R6 gpl40 trimer was incubated with 0 (no detergent control), 0.1, 0.05, 0.01, 0.001, or 0.0001% Tween® 20 and analyzed by BN-PAGE and Coomassie G-250 stain. Arrows point to HMW aggregate and trimer species. M stands for the 669k thyroglobulin and 440k ferritin molecular weight protein standards. (B) Time course: Purified KNH1 144 SOSIP R6 gpl40 trimer was incubated with Tween® 20 for 5 min (left panel) or 10 min (right panel). Trimer was either untreated (- lane) or Tween® 20 treated (+ lane). Arrows indicate trimer and
HMW aggregate bands. (C) Temperature effect: Purified KNH1 144 SOSIP R6 gpl40 trimer was either untreated (- lane) or treated with Tween® 20 at room temperature (RT) or 37°C. Reactions were analyzed by BN-PAGE and Coomassie G-250 stain. Arrows indicate HMW aggregate and trimer proteins. (D) Tween® 20 effect on HMW aggregate and dimer fractions: A preparation composed predominantly of HMW aggregate (> 80%) was untreated (left panel, - lane), or incubated with Tween® 20 (left panel, + lane), and analyzed by BN-PAGE and Coomassie G-250 stain. Solid arrows indicate HMW aggregate and trimer proteins. Preparations composed of HMW aggregate, dimers and monomers were untreated (right panel, - lane) or incubated with Tween® 20 (right panel, + lane) and analyzed by BN-PAGE and Coomassie G-250 stain. Arrows on the right hand side point to aggregate, trimer, dimer and monomer species.

FIGURE 3: Size Exchange Chromatography (SEC) analysis of KNH1 144 SOSIP R6 gpl40 trimer. KNH1 144 SOSIP R6 gpl40 trimer was resolved on a Superdex 200 10/300 GL column in TN-500 buffer containing 0.05% Tween® 20 (TNT-500). The A$_{280}$ protein profile of the run is shown in the middle panel. Fractions B7-C3 from the run were analyzed by BN-PAGE, followed by silver stain (bottom panel). Arrows to the side of the BN-PAGE image point to the trimer. The vertical arrow in the BN-PAGE indicates the peak signal of the trimer in fraction B12. The arrow in the middle chromatograph corresponds to fraction B12.

FIGURE 4: Effect of Tween® 20 treatment on KNH1 144 SOSIP R6 HMW aggregate antigenicity. Lectin ELISA of untreated and Tween® 20 treated KNH1 144 SOSIP R6 HMW aggregate: Untreated or Tween® 20-treated HMW aggregate were bound to GNA lectin coated ELISA plates and probed with 2Gl2, b6, bl2, CD4-IgG2, and HIVIg. The panels represent their respective binding curves. Antibody affinity to the untreated HMW aggregate is represented by the curve having diamond lines. Affinity to the Tween® 20 treated HMW aggregate is represented by curve having square lines. The Y-axis represents the colorimetric signal at OD492 and the X-axis represents antibody concentration in [ug/ml]. Lectin ELISA of untreated and Tween® 20-treated KNH1 144 SOSIP R6 gpl40 trimer: Untreated or Tween® 20 treated trimer (containing 10-15% HMW aggregate) were bound to GNA lectin coated ELISA plates and probed with 2Gl2, b6, bl2, and CD4-IgG2. The panels represent their respective binding curves. Antibody affinity to the untreated trimer is represented by the curve having diamond lines. Affinity to the Tween® 20 treated trimer is represented by the curve having square lines. The Y-axis represents the colorimetric signal at OD492 and the X-axis represents antibody concentration in [ug/ml].
FIGURE 5: Effect of Tween® 20 treatment on KNHl 44 SOSIP R6 gpl40 trimer binding to DEAE anion exchange column. Purified KNHl 144 SOSIP R6 gpl40 trimer, spiked with alpha-2 macroglobulin (a₂M) contaminant, was either untreated or treated with Tween® 20. Following treatment, sample was applied over an anion exchange column (DEAE HiTrap FF 1 ml column) (Load). Flow through (FT) fractions were collected and the column was washed (Wash). The column was eluted (Elution) and fractions were analyzed over BN-PAGE, followed by Coomassie G-250 stain. The top panel shows fractions analyzed from the untreated control trimer DEAE application. The bottom panel shows fractions analyzed from the Tween® 20 treated trimer DEAE application. Arrows point to trimer and a₂M contaminant proteins. M stands for the 669k thyroglobulin and 440k ferritin molecular weight protein standards. Asterisks highlight the fraction where the trimer is found.

FIGURE 6: Negative stain electron micrographs of KNHl144 SOSIP R6 gpl40 trimers. KNHl 144 SOSIP R6 gpl40 trimers were analyzed by negative stain electron microscopy. Bar = 50nm.

FIGURE 7: SEC analysis of KNHl144 gpl20 monomer: KNHl 144 gpl20 monomer was resolved on a Superdex 200 10/300 GL column in TN-500 buffer. The top chromatograph shows its A₂₈₀ protein profile of the run. As a control, JR-FL gpl20 monomer was resolved in a similar manner and its A₂₈₀ protein profile is displayed in the bottom chromatograph. The observed retention times for both monomers and their apparent calculated molecular weights are indicated.

FIGURE 8: Tween® 20 effect on a₂M: Purified a₂M was incubated with Tween® 20 (+ lane) or waa untreated (- lane). Reactions were analyzed by BN-PAGE and Coomassie stain. Arrow indicates a₂M band.

FIGURE 9: Amino acid sequence (SEQ ID NO:1) of modified gpl40 the HIV-I KNHl 144 isolate.

FIGURE 10: Nucleic acid sequence (SEQ ID NO:7) and amino acid sequence (SEQ ID NO:4) of HIV-I 5768.4 isolate.

FIGURES HA and HB: Gel analysis (A) BN-PAGE and (B) SDS-PAGE of purified trimer product using the purification method described in Experimental Details III.
FIGURE 12: Gel analysis (BN-PAGE/Silver) of purified trimer using the purification method described in Experimental Details II (fifth lane) and using the purification method described in Experimental Details III (third lane). Molecular weight marker (first lane).

FIGURES 13A and 13B: Immunoprecipitation (IP) of KNHI 144 SOSIP.R6 using ARP3110 probing antibody (A) using the purification method described in Experimental Details III and (B) using the purification method described in Experimental Details II.

FIGURES 14A and 14B: Western Blot analysis of KNHI 144 SOSIP.R6 using ARP3110 probing antibody (A) using the purification method described in Experimental Details III and (B) using the purification method described in Experimental Details II.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

As used in this application, except as otherwise expressly provided herein, each of the following terms shall have the meaning set forth below.

The following standard abbreviations are used throughout the specification to indicate specific amino acids: A=ala=alanine; R=arg=arginine; N=asn=asparagine; D=asp=aspartic acid; C=cys=cysteine; Q=gln=glutamine; E=glu=glutamic acid; G=gly=glycine; H=his=histidine; I=ile=isoleucine; L=leu=leucine; K=lys=lysine; M=met=methionine; F=phe=phenylalanine; P=pro=proline; S=ser=serine; T=thr=threonine; W=trp=tryptophan; Y=tyr=tyrosine; V=val=valine; B=asn=asparagine or aspartic acid; Z=glx=glutamine or glutamic acid.

An "A5I 1C mutation" refers to a point mutation of amino acid 511 in the HIV-1 KNH1 144 isolate gp120 from alanine to cysteine. Because of sequence and sequence numbering variability among different HIV strains and isolates, it will be appreciated that this amino acid will not be at position 511 in all other HTV isolates. For example, in HIV-1JR-FL the corresponding amino acid is A492 (Genbank Accession No. U63632), in HIV-1HXB2 the corresponding amino acid is A501 (Genbank Accession No. AAB50262) and in HIV-1NL4-3 it is A499 (Genbank Accession No. AAA44992). The amino acid may also be an amino acid other than alanine which has similar polarity or charge characteristics, for example. This invention encompasses cysteine mutations in such amino acids, which can be readily identified in other HIV isolates by those skilled in the art. This invention encompasses the replacement of such amino acids by cysteine, as may be readily identified in other HIV isolates by those skilled in the art.
"157IP" refers to a point mutation wherein the isoleucine residue at position 571 of a polypeptide chain is replaced by a proline residue.

A "T617C mutation" refers to a point mutation of amino acid 617 in HTV-I KNHI 144 isolate gp41 ectodomain from threonine to cysteine. Because of sequence and sequence numbering variability among different HIV strains and isolates, it will be appreciated that this amino acid will not be at position 617 in all other HIV isolates. For example, in HIV-I \(_{\text{JR-FL}}\) the corresponding amino acid is T596 (Genbank Accession No. U63632), in HIV-I \(_{\text{HXB2}}\) the corresponding amino acid is T605 (Genbank Accession No. AAB50262) and in HIV-1 \(_{\text{NL4-3}}\) the corresponding amino acid is T603 (Genbank Accession No. AAA44992). The amino acid may also be an amino acid other than threonine which has similar polarity or charge characteristics, for example. This invention encompasses cysteine mutations in such amino acids, which can be readily identified in other HIV isolates by those skilled in the art. This invention encompasses the replacement of such amino acids by cysteine, as may be readily identified in other HIV isolates by those skilled in the art.

An "A519C mutation" refers to a point mutation of amino acid 519 in HIV-I 5768.4 isolate gp120 from alanine to cysteine. Because of sequence and sequence numbering variability among different HIV strains and isolates, it will be appreciated that this amino acid will not be at position 519 in all other HIV isolates. For example, in HIV-I \(_{\text{JR-FL}}\) the corresponding amino acid is A492 (Genbank Accession No. U63632), in HIV-I \(_{\text{HXB2}}\) the corresponding amino acid is A501 (Genbank Accession No. AAB50262) and in HIV-1 \(_{\text{NL4-3}}\) it is A499 (Genbank Accession No. AAA44992). The amino acid may also be an amino acid other than alanine which has similar polarity or charge characteristics, for example. This invention encompasses cysteine mutations in such amino acids, which can be readily identified in other HIV isolates by those skilled in the art. This invention encompasses the replacement of such amino acids by cysteine, as may be readily identified in other HIV isolates by those skilled in the art.

"1579P" refers to a point mutation wherein the isoleucine residue at position 579 of a polypeptide chain is replaced by a proline residue.

A "T625C mutation" refers to a point mutation of amino acid 625 in HIV-I 5768.4 isolate gp41 ectodomain from threonine to cysteine. Because of sequence and sequence numbering variability among different HIV strains and isolates, it will be appreciated that this amino acid will not be at position 625 in all other HIV isolates. For example, in HIV-I \(_{\text{JR-FL}}\) the corresponding amino acid is T596 (Genbank Accession No. U63632), in HIV-I \(_{\text{HXB2}}\) the corresponding amino acid is T605
(Genbank Accession No. AAB50262) and in HIV-IN the corresponding amino acid is T603 (Genbank Accession No. AAA44992). The amino acid may also be an amino acid other than threonine which has similar polarity or charge characteristics, for example. This invention encompasses the replacement of such amino acids by cysteine, as may be readily identified in other HIV isolates by those skilled in the art.

"HIV" refers to the human immunodeficiency virus. HIV shall include, without limitation, HIV-1. The human immunodeficiency virus (HIV) may be either of the two known types of HIV (HIV-I or Hrv2). The HTV-I virus may represent any of the known major subtypes (Classes A, B, C, D E, F, G and H) or outlying subtype (Group O).

"gpl40 envelope" refers to a protein having two disulfide-linked polypeptide chains, the first chain comprising the amino acid sequence of the HIV gpl20 glycoprotein and the second chain comprising the amino acid sequence of the water-soluble portion of HIV gp41 glycoprotein ("gp41 portion"). HIV gpl40 protein includes, without limitation, proteins wherein the gp41 portion comprises a point mutation such as I579P. gp40 envelope comprising such mutation is also referred to as "HIV SOS gpl40", as well as "HIV gplRO monomer" or "SOSEP gpl40".

"gp41" includes, without limitation, (a) whole gp41 including the transmembrane and cytoplasmic domains; (b) gp41 ectodomain (gp4IEC); (c) gp41 modified by deletion or insertion of one or more glycosylation sites; (d) gp41 modified so as to eliminate or mask the well-known immunodominant epitope; (e) a gp41 fusion protein; and (f) gp41 labeled with an affinity ligand or other detectable marker. As used herein, "ectodomain" means the extracellular region of a transmembrane protein exclusive of the transmembrane spanning and cytoplasmic regions.

Embodiments of the Invention

This invention provides a process of recovering trimers of a retroviral envelope (Etv) protein from a preparation comprising trimers of the retroviral Env protein and high molecular weight aggregates thereof which comprises treating the preparation with a non-ionic detergent so as to thereby convert the high molecular weight aggregates into trimers, and recovering the trimers from the preparation so treated. In one embodiment, the retroviral Env protein is an HIV Env protein. In another embodiment, the retroviral Env protein is an HIV-I or HIV-2 Env protein.
In one embodiment, the above process further comprises formulating the recovered trimers with a pharmaceutically acceptable carrier, excipient, or diluent.

This invention also provides a process of recovering trimers of a retroviral envelope (Env) protein from a preparation comprising trimers of the Env protein and high molecular weight aggregates thereof which comprises: (a) subjecting the preparation to lectin chromatography to obtain a trimer-enriched eluate in which aggregate content is reduced; (b) applying the eluate of step (a) to a first diethylaminoethyl (DEAE) sepharose chromatography column in the absence of non-ionic detergent to obtain elution fractions further enriched in trimers; (c) applying trimer-enriched elution fractions from the first DEAE sepharose chromatography column of step (b) to a second DEAE sepharose chromatography column in the presence of non-ionic detergent; and (d) collecting the flow through and wash fractions from the second DEAE sepharose chromatography column in the presence of non-ionic detergent, so as to thereby recover the trimers of the retroviral Env protein. In one embodiment, the retroviral Env protein is an HIV Env protein. In another embodiment, the retroviral Env protein is an HIV-1 or HIV-2 Env protein.

In one embodiment of the above process, in step (a), the preparation is a concentrated cell culture fluid. In another embodiment, in step (a), the preparation is subjected to ammonium sulfate precipitation prior to the lectin chromatography. In another embodiment, in step (a), column fractions enriched in trimers are eluted through a linear gradient in PBS to 1.0 M methyl-a-D-mannopyranoside (MMP) in ten column volumes. In yet another embodiment, in step (b), fractions further enriched in trimers are eluted from the DEAE chromatography column through a linear gradient to 20 mM Tris, 0.3 M NaCl, pH 8.0 in ten column volumes. In one embodiment, in step (c), the fractions further enriched in trimers are eluted from the second DEAE chromatography column through a linear gradient to 20 mM Tris, 0.3 M NaCl, pH 8.0 in ten column volumes. In one embodiment, the wash fractions of step (d) comprise buffer which comprises 20 mM Tris and 75 mM NaCl, pH 7.5.

This invention further provides a process of recovering trimers of a retroviral envelope (Env) protein from a preparation comprising trimers of the retroviral Env protein and high molecular weight aggregates thereof, which comprises: (a) subjecting the preparation to lectin affinity chromatography to obtain an eluate enriched in trimers and essentially separated from monomer and dimer aggregates and macroglobulin; and (b) subjecting the eluate of step (a) to a first diethylaminoethyl sepharose chromatography column in the absence of non-ionic detergent so as
to further remove aggregates in the flow through; and (c) subjecting the eluate of step (b) to a second diethylaminoethyl sepharose chromatography column in the presence of non-ionic detergent so as to recover the trimers of the retroviral Env protein. In one embodiment, the retroviral Env protein is an HIV Env protein. In another embodiment, the retroviral Env protein is an HIV-I or HIV-2 Env protein.

In one embodiment of the above process, in step (a), the preparation is a concentrated cell culture fluid. In another embodiment, in step (a), the preparation is subjected to ammonium sulfate precipitation prior to the lectin chromatography. In yet another embodiment, the trimers are recovered in the presence of the non-ionic detergent so as to maintain trimer stability upon subsequent storage. In one embodiment, the non-ionic detergent is a polyethylene type detergent.

In another embodiment, the polyethylene type detergent is a poly(oxyethylene) sorbitan monolaureate. In another embodiment, the poly(oxyethylene) sorbitan monolaureate is poly(oxyethylene) (20) sorbitan monolaureate. In one embodiment, the polyethylene type detergent is poly(oxyethylene) sorbitan monooleate.

In one embodiment, the Env protein comprises consecutive amino acids having a sequence corresponding to that of a naturally occurring Env protein of an HIV-I subtype A isolate or a mutant derived therefrom. In one embodiment, the HIV-I subtype A isolate is KNHI 144.

In one embodiment, the Env protein comprises consecutive amino acids having a sequence corresponding to that of a naturally occurring Env protein of an HIV-I subtype B isolate or a mutant derived therefrom. In one embodiment, the HIV-I subtype B isolate is 5768.4. In another embodiment of the above process, steps (a) through (d) are carried out at room temperature. In one embodiment, steps (a) through (c) are carried out at room temperature. In one embodiment, the non-ionic detergent present is at a concentration of 0.001% to 1% by weight of the preparation. In another embodiment, the concentration of the non-ionic detergent is from 0.01% to 1% by weight. In one embodiment, the concentration of the non-ionic detergent is from 0.025% to 1% by weight. In another embodiment, the concentration of the non-ionic detergent is from 0.01% to 0.5% by weight. In another embodiment, the concentration of the non-ionic detergent is from 0.01% to 0.1% by weight. In one embodiment, the concentration of the non-ionic detergent is from 0.01% to 0.05% by weight. In another embodiment, the concentration of the non-ionic detergent is 0.05% by weight.
In one embodiment of the above process, prior to treatment with the non-ionic detergent, the preparation comprises greater than 10% high molecular weight aggregates. In another embodiment, prior to treatment with the non-ionic detergent, the preparation comprises 10-40% high molecular weight aggregates. In yet another embodiment, prior to treatment with the non-ionic detergent the preparation comprises greater than 70% high molecular weight aggregates. The Env protein comprises (a) a first polypeptide which comprises consecutive amino acids, the sequence of which corresponds to the sequence of a modified gpl20 envelope polypeptide portion of a gpl40 envelope of an HIV-I KNH1 144 isolate, or a quasi-species thereof; and (b) a second polypeptide which comprises consecutive amino acids, the sequence of which corresponds to the sequence of a modified gp41 ectodomain polypeptide portion of the gpl40 envelope of the HIV-I KNH1 144 isolate or such quasi-species thereof, the sequence of said modified gpl20 envelope polypeptide portion and said modified gp41 ectodomain polypeptide portion of said HIV-I KNH1 144 isolate being as set forth in SEQ ID NO:2 and SEQ ID NO:3, respectively, said modified gpl20 envelope polypeptide portion comprising a cysteine at amino acid position 511 and said modified gp41 ectodomain polypeptide portion comprising a cysteine at amino acid position 617 and a proline at amino acid position 571, wherein (i) the amino acid positions are numbered by reference to SEQ ID NO:1, (ii) the modified gpl20 envelope polypeptide portion further comprises a mutated furin recognition sequence, and (iii) the modified gpl20 polypeptide portion and the modified gp41 ectodomain polypeptide portion are bound to one another by a disulfide bond between the cysteine at amino acid position 511 and the cysteine at amino acid position 617.

In one embodiment, the Env protein comprises (a) a first polypeptide which comprises consecutive amino acids, the sequence of which corresponds to the sequence of a modified gpl20 envelope polypeptide portion of a gpl40 envelope of an HIV-I 5768.4 isolate, or a quasi-species thereof; and (b) a second polypeptide which comprises consecutive amino acids, the sequence of which corresponds to the sequence of a modified gp41 ectodomain polypeptide portion of the gpl40 envelope of the HW-I 5768.4 isolate or such quasi-species thereof, the sequence of said modified gpl20 envelope polypeptide portion and said modified gp41 ectodomain polypeptide portion of said HIV-I 5768.4 isolate being as set forth in SEQ ID NO:5 and SEQ ID NO:6, respectively, said modified gpl20 envelope polypeptide portion comprising a cysteine at amino acid position 519 and said modified gp41 ectodomain polypeptide portion comprising a cysteine at amino acid position 625 and a proline at amino acid position 579, wherein (i) the amino acid positions are numbered by reference to SEQ ID NO:4, (ii) the modified gpl20 envelope polypeptide portion further comprises a mutated furin recognition sequence, and (iii) the
modified gpl20 polypeptide portion and the modified gp41 ectodomain polypeptide portion are bound to one another by a disulfide bond between the cysteine at amino acid position 519 and the cysteine at amino acid position 625. In one embodiment, the recovering further comprises purifying the trimers. In one embodiment, at least 50 µg of trimers are recovered from the preparation. In another embodiment, the above process further comprises formulating the recovered trimers with a pharmaceutically acceptable carrier, excipient, or diluent.

In the above-described trimer purification methods/processes, at least 50 µg of trimers are recovered from the starting preparation. In an embodiment, the above processes further comprise formulating the recovered trimers with a pharmaceutically acceptable carrier, excipient, or diluent.

This invention also provides a process of recovering trimers of a retroviral envelope (Env) protein from a preparation comprising trimers of the Env protein and high molecular weight aggregates thereof which comprises subjecting the preparation to size exclusion chromatography in the presence of a non-ionic detergent so as to convert the high molecular weight aggregates into trimers, and recovering the trimers from the preparation subjected to the chromatography. In one embodiment, the retroviral Env protein is an HIV Env protein. In another embodiment, the retroviral Env protein is an HIV-1 or HIV-2 Env protein.

In one embodiment, the process comprises subjecting the preparation to size exclusion chromatography, and then subjecting the resulting preparation to anion exchange chromatography before recovering the trimers.

In another embodiment, the processes of the invention provide trimers which are recovered in the presence of non-ionic detergent so as to maintain trimer stability upon subsequent storage. The trimers in non-ionic detergent according to this invention are stable for days, weeks and months, e.g., greater than one week, greater than two weeks, greater than one month, greater than two months, or greater than six months to years, for example, −4°C-25°C, at room temperature (~16°C-25°C), or frozen. In one embodiment, the non-ionic detergent is a polyethylene type detergent. In one embodiment, the polyethylene type detergent is a poly (oxyethylene) sorbitan monolaureate. In another embodiment, the poly (oxyethylene) sorbitan monolaureate is poly (oxyethylene) sorbitan monolaureate. In yet another embodiment, the polyethylene type detergent is poly (oxyethylene) sorbitan monooleate.
In one embodiment, the Env protein comprises consecutive amino acids having a sequence corresponding to that of a naturally occurring Env protein of an HIV-I subtype A isolate, a mutant derived therefrom, or a quasi-species thereof. In another embodiment, the HIV-I subtype A isolate is KNHI 144.

In one embodiment, the Env protein comprises consecutive amino acids having a sequence corresponding to that of a naturally occurring Env protein of an HIV-I subtype B isolate, a mutant derived therefrom, or a quasi-species thereof. In another embodiment, the HIV-I subtype B isolate is 5768.4.

This invention further provides a process of recovering trimers of a retroviral envelope (Env) protein from a preparation comprising trimers of the Env protein and high molecular weight aggregates thereof which comprises (a) subjecting the preparation to size exclusion chromatography to obtain eluted fractions; (b) subjecting the fractions of step (a) to anion exchange chromatography; and (c) treating the column fractions of step (b) with a non-ionic detergent, so as to thereby recover the trimers of the Env protein. In one embodiment, the process further comprises re-subjecting the column fractions of step (b) to anion exchange chromatography prior to step (c). In another embodiment, approximately 30-40% more trimers are recovered following re-subjecting the fractions of step (b) to anion exchange chromatography. In another embodiment, the process further comprises re-subjecting the fractions of step (c) to anion exchange chromatography (e.g., DEAE anion exchange chromatography) to remove non-aggregate contaminants. In another embodiment, the size exclusion chromatography of step (a) is performed in the presence of a non-ionic detergent. In another embodiment, the retroviral Env protein is an HIV Env protein. In yet another embodiment, the retroviral Env protein is an HIV-I or HIV-2 Env protein.

This invention also provides a process of recovering trimers of a retroviral envelope (Env) protein from a preparation comprising trimers of the retroviral Env protein and high molecular weight aggregates thereof which comprises (a) subjecting the preparation to lectin affinity chromatography to obtain elution fractions; and (b) subjecting the fractions of step (a) to size exclusion chromatography in the presence of a non-ionic detergent so as to thereby recover the trimers of the Env protein.

This invention further provides a process of recovering trimers of a retroviral envelope (Env) protein from a preparation comprising trimers of the Env protein and high molecular weight aggregates thereof which comprises (a) subjecting the preparation to size exclusion chromatography to obtain eluted fractions; (b) subjecting the fractions of step (a) to anion exchange chromatography; and (c) treating the column fractions of step (b) with a detergent, so as to thereby recover the trimers of the Env protein.
aggregates thereof which comprises (a) subjecting the preparation to lectin affinity chromatography to obtain fractions comprising monomers, dimers, and trimers of the Env protein; (b) subjecting the fractions of step (a) to size exclusion chromatography to obtain fractions comprising dimers and trimers of the Env protein; (c) subjecting the fractions of step (b) to anion exchange chromatography to obtain fractions in which trimers of the Env protein are separated from dimers; and (d) treating trimer-containing fractions of step (c) with non-ionic detergent, so as to thereby recover the trimers of the Env proteins. In one embodiment, the non-ionic detergent is a polyethylene type detergent. In another embodiment, the polyethylene type detergent is poly (oxyethylene) sorbitan monolaureate. In yet another embodiment, the poly (oxyethylene) sorbitan monolaureate is poly (oxyethylene) (20) sorbitan monolaureate. In another embodiment, the polyethylene type detergent is poly (oxyethylene) sorbitan monooleate.

In one embodiment, the process further comprises re-subjecting the fractions of step (c) to anion exchange chromatography following step (c) and prior to step (d). In another embodiment, approximately 30-40% more trimers are recovered following re-subjecting the fractions of step (c) to anion exchange chromatography.

In yet another embodiment, the process further comprises re-subjecting the fractions of step (d) to anion exchange chromatography to remove non-aggregate contaminants. In one embodiment, the retroviral Env protein is an HIV Env protein. In another embodiment, the retroviral Env protein is an HIV-I or HIV-2 Env protein.

In one embodiment of the processes of the invention, prior to treatment with the non-ionic detergent, the preparation comprises greater than 10% high molecular weight aggregates.

In another embodiment, prior to treatment with the non-ionic detergent, the preparation comprises 10-40% high molecular weight aggregates. In yet another embodiment, prior to treatment with the non-ionic detergent, the preparation comprises greater than 70% high molecular weight aggregates.

In one embodiment of the processes of the invention, the non-ionic detergent is present at a concentration of between 0.001% to 1% by weight of the preparation. In another embodiment, the concentration of non-ionic detergent is from 0.01% to 1% by weight of the preparation. In another embodiment, the concentration of non-ionic detergent is from 0.025% to 1% by weight of the preparation. In yet another embodiment, the concentration of non-ionic detergent is from 0.01% to 0.5% by weight of the preparation. In a further embodiment, the concentration of non-
ionic detergent is from 0.01% to 0.1% by weight of the preparation. In another embodiment, the concentration of non-ionic detergent is from 0.01% to 0.05%. In yet another embodiment, the concentration of non-ionic detergent is 0.05%.

In one embodiment, the retroviral Env protein is an HIV Env protein. In another embodiment, the retroviral Env protein is an HIV-1 or HIV-2 Env protein. In one embodiment, the Env protein comprises consecutive amino acids having a sequence corresponding to that of a naturally occurring Env protein of an HIV-1 subtype A isolate or a mutant derived therefrom. In one embodiment, the HIV-I subtype A isolate is KNHi 144. In another embodiment, the Env protein comprises consecutive amino acids having a sequence corresponding to that of a naturally occurring Env protein of an HIV-I subtype B isolate or a mutant derived therefrom. In one embodiment, the HIV-I subtype B isolate is 5768.4.

This invention also provides processes described above, which further comprise formulating the recovered trimers with a pharmaceutically acceptable carrier, excipient, or diluent.

This invention also provides a process wherein the Env protein comprises (a) a first polypeptide which comprises consecutive amino acids, the sequence of which corresponds to the sequence of a modified gp120 envelope polypeptide portion of a gp140 envelope of an HIV-1 KNHi 144 isolate, or a quasi-species thereof; and (b) a second polypeptide which comprises consecutive amino acids, the sequence of which corresponds to the sequence of a modified gp41 ectodomain polypeptide portion of the gp140 envelope of the HIV-I KNHi 144 isolate or such quasi-species thereof, the sequence of said modified gp120 envelope polypeptide portion and said modified gp41 ectodomain polypeptide portion of said HIV-I KNHi 144 isolate being as set forth in SEQ ID NO:2 and SEQ ID NO:3, respectively, said modified gp120 envelope polypeptide portion comprising a cysteine at amino acid position 511 and said modified gp41 ectodomain polypeptide portion comprising a cysteine at amino acid position 617 and a proline at amino acid position 571, wherein (i) the amino acid positions are numbered by reference to SEQ ID NO:1, (ii) the modified gp120 envelope polypeptide portion further comprises a mutated furin recognition sequence, and (iii) the modified gp120 polypeptide portion and the modified gp41 ectodomain polypeptide portion are bound to one another by a disulfide bond between the cysteine at amino acid position 511 and the cysteine at amino acid position 617. In one embodiment, the cysteine at position 511 is the result of an A511C mutation. In another embodiment, the cysteine at position 617 is the result of a T617C mutation. In yet another embodiment, the proline at position 571 is the result of an I571P mutation.
This invention further provides a process wherein the Env protein comprises (a) a first polypeptide which comprises consecutive amino acids, the sequence of which corresponds to the sequence of a modified gpl20 envelope polypeptide portion of a gpl40 envelope of an HTV-I S768.4 isolate, or a quasi-species thereof; and (b) a second polypeptide which comprises consecutive amino acids, the sequence of which corresponds to the sequence of a modified gp41 ectodomain polypeptide portion of the gpl40 envelope of the HIV-I 5768.4 isolate or such quasi-species thereof, the sequence of said modified gpl20 envelope polypeptide portion and said modified gp41 ectodomain polypeptide portion of said HIV-I 5768.4 isolate being as set forth in SEQ ID NO:5 and SEQ ID NO:6, respectively, said modified gpl20 envelope polypeptide portion comprising a cysteine at amino acid position 519 and said modified gp41 ectodomain polypeptide portion comprising a cysteine at amino acid position 625 and a proline at amino acid position 579, wherein (i) the amino acid positions are numbered by reference to SEQ ID NO:4, (ii) the modified gpl20 envelope polypeptide portion further comprises a mutated furin recognition sequence, and (iii) the modified gpl20 polypeptide portion and the modified gp41 ectodomain polypeptide portion are bound to one another by a disulfide bond between the cysteine at amino acid position 519 and the cysteine at amino acid position 625. In one embodiment, the cysteine at position 519 is the result of a R519C mutation. In another embodiment, the cysteine at position 625 is the result of a T625C mutation. In yet another embodiment, the proline at position 579 is the result of an I579P mutation. In one embodiment, the process further comprises purifying the trimers so recovered.

This invention is illustrated in the Experimental Details section which follows. This section is set forth to aid in an understanding of the invention but is not intended to, and should not be construed to limit in any way the invention as set forth in the claims which follow thereafter.

**EXPERIMENTAL DETAILS**

**INTRODUCTION**

The present invention as described herein encompasses the purification and biochemical characterization of a KNHI 144 SOSIP R6 gpl40, derived from a contemporary East African subtype A HIV-I primary isolate, using novel methodologies that are different from previously implemented purification procedures. The purified KNHI 144 SOSIP R6 gpl40 is a trimer based on BN-PAGE and size exclusion chromatography (SEC) analyses. The present invention is directed to novel methods involving the use of non-ionic detergents, such as Tween® 20, to recover homogeneous trimeric forms of the KNHI 144 SOSIP R6 Env gpl40 protein from aggregates thereof. Trimers are recovered (also termed "collapsed" or "converted") from
aggregates (of monomers, dimers and trimers) at greater than 90-98% purity, typically 100% purity using non-ionic detergent in accordance with this invention. The present invention also provides novel insights into the nature of the aggregate species. The effects of non-ionic detergent, e.g., Tween® 20, treatment on the antigenic properties of KNHl 144 SOSIP R6 gpl40 aggregates and trimers were examined. Finally, digital imaging based on negative stain electron microscopy was perform and reveals the structure of purified KNHl 144 SOSIP R6 gpl40 as trimeric oligomers.

Additionally, as described hereibelow, the present invention is further directed to novel methods involving the use of non-ionic detergents, such as Tween® 20, to recover homogeneous trimeric forms of the subtype B 5768.4 SOSIP R6 Env gpl40 protein from aggregates thereof. Trimers are recovered (also termed "collapsed") from aggregates at greater than 90-98% purity, typically 100% purity using non-ionic detergent.

15 MATERIALS AND METHODS
Subtype A KNHl 144 SOSIP R6 transfection and expression:
The KNHl 144 SOSIP R6 envelope and furin DNA plasmids were as described. For a typical 8 L preparation, HEK 293T cells were seeded in triple flasks at a density of 2.5 x 10^7 cells/flask and cultured in DMEM/10% FBS/1% pen-strep with 1% L-glutamine 24 hours prior to transfection. On the day of transfection, 270 ug of KNHl 144 SOSIP R6 envelope DNA was mixed with 90 ug of Furin protease DNA plasmid (per flask) in Opti-MEM. Polyethyleneimine (PEI) was added stepwise (2 mg PEI: 1 mg total DNA) and vortexed immediately in between each addition. The PEI/DNA complex solutions were incubated for 20 minutes at room temperature. Complexes were then added to the flasks and incubated for 6 hours at 32°C, 5% CO₂. The cells were then washed with warmed PBS and then incubated in exchange media (DMEM/ 0.05% BSA/1% pen-strep) for 48 hours at 32°C, 5% CO₂. After the 48 hour incubation, the supernatants were collected and a cocktail of protease inhibitors was added to minimize protein degradation. Harvested supernatants were then clarified by filtration through a 0.45um filter and concentrated to 53X. Expression of KNHl 144 gpl20 monomer has been previously described (1) and typically, 1-2 L of cell culture supernatants from transfected cells were harvested. Supernatants were clarified by filtration and stored at -80°C without any concentration prior to purification.
Purification of KNHl 144 SOSIP R6 f_pl40 and gpl20 :

KNHl 144 SOSIP R6 gpl40 trimer was purified via a four step process starting with an ammonium sulfate precipitation followed by lectin affinity, size exclusion and ion-exchange chromatography. 53X concentrated cell culture supernatant was precipitated with an equal volume of 3.8 M ammonium sulfate to remove contaminant proteins (with the major contaminant being α-2-macroglobulin). The ammonium sulfate was added with constant stirring with a stir bar and then was immediately centrifuged at 4000 rpm, 4°C for 45 minutes. The resulting supernatant was diluted 4-fold with PBS, pH 7.25, and was filtered using a 0.45 um vacuum filter. The sample was then loaded at 0.5-0.8 ml/min onto a Galanthus nivalis (GNA) lectin (Vector Laboratories, Burlingame, CA) column equilibrated with PBS- pH 7.25. Once the load was finished, the column was washed with PBS pH 7.25 until OD_{280} reached baseline, followed by a second wash with 0.5 M NaCl PBS pH 7.25 at 1 ml/min in order to remove contaminant proteins (mainly BSA). The column was then eluted with 1 M MMP PBS pH 7.25 starting with flowing one half CV through the column at 0.3 ml/min and pausing the purification for a 1 hour incubation in MMP elution buffer. Following the incubation, the flow was restarted at 0.3 ml/min and 0.5-1 ml fractions were collected. All peak fractions were then pooled and concentrated to a final volume of 1 ml using a Vivaspin 100,000 MWCO concentrator (Vivascience, Edgewood, NY) centrifuged at 1000 x g. The concentrated lectin elution was applied over a Superdex 200 SEC column (GE Healthcare, Piscataway, NJ) equilibrated in 20 mM Tris pH 8, 200 mM NaCl (TN-200), injecting 0.5 ml of sample per run and was resolved at 0.4 ml/min, collecting 0.4 ml fractions. The fractions were analyzed by BN-PAGE using a 4-12% Bis-Tris NuPAGE gel (Invitrogen, Carlsbad, CA) (10). All trimer containing fractions were pooled and diluted to 75 mM NaCl with 20mM Tris pH 8. The diluted SEC pool was then applied over a 1 ml HiTrap DEAE FF column (GE Healthcare), equilibrated in 20 mM Tris pH 8, 75 mM NaCl (TN-75). The diluted SEC pool was loaded at 0.5 ml/min. The column was washed with TN-75 at 1 ml/min until the OD_{280} reached baseline. The column was then eluted with 20 mM Tris, 300 mM NaCl pH 8 at 1 ml/min, collecting 0.5 ml fractions.

To maximize trimer yield, the flow-through fraction from the DEAE column was re-applied over the column (equilibrated in TN-75) and typically 20-30% or 30-40% more trimer was recovered in this manner. The fractions were analyzed by BN-PAGE and by reducing and non-reducing SDS-PAGE. Western blot analysis on non-reduced SDS-PAGE gel was performed with the ARP31 19 monoclonal antibody. The trimer containing fractions were pooled and trimer concentration was determined through densitometry on a reducing SDS-PAGE gel using IR-FL gp120 as a standard.
KNHl 144 gpl20 monomer:
Unconcentrated cell culture supernatants containing secreted gpl20 monomer were applied directly over a GNA lectin column equilibrated in 20 mM imidazole pH 7.1 at 1-2 ml/min. Following adsorption, the column was washed with a high salt (PBS containing 1 M NaCl, pH 7.1) wash, followed by a low salt (20 mM imidazole pH 7.1) wash. The column was eluted with 1 M MMP in 20 mM imidazole, 0.2 M NaCl pH 7.1. Peak fractions were pooled and diluted with 20 mM imidazole, pH 7.1, thirteen-fold to a final buffer concentration of 20 mM imidazole, pH 7.1, 15 mM NaCl. The diluted GNA elution was applied over 1 ml HiTrap Q Sepharose FF (GE Healthcare) equilibrated in 20 mM imidazole, pH 7.1. Following binding, the column was washed with 20 mM imidazole, pH 7.1, and was eluted with 20 mM imidazole, 0.2 M NaCl, pH 7.1. The Q elutions were pooled and concentrated and applied over a Superdex 200 column equilibrated in PBS in 0.5 ml volumes and resolved at 0.4 ml/min. Peak fractions were analyzed by 4-12% Bis-tris gels (Invitrogen), followed by Coomassie staining. Fractions containing gpl20 were pooled and quantified as described above for the SOSIP R.6 gpl40 trimers and stored at -80°C.

Tween® 20 Aggregate "conversion"/"collapse" experiments:

**Tween® 20 Dose effect:** 1 ug of purified KNHI 144 SOSIP R6 trimer was incubated with varying concentrations of Tween® 20 (polyoxyethylene sorbitan monolaurate) ranging from 0 to 0.0001 % (v/v) and incubated for 1 hour at room temperature. Following incubation, samples were analyzed by BN-PAGE as described above.

**Kinetics of Tween® 20 effect:** To ascertain the early kinetics of the Tween® 20 effect on aggregate, 1 ug of purified KNHI 144 SOSIP R6 trimer was incubated with Tween® 20 at a final concentration of 0.05 % (v/v) for 5 minutes and for 10 minutes. A no-detergent control was included separately for each timepoint.

**Temperature dependance on Tween® 20 effect:** To determine if temperature affected the ability of Tween® 20 to recover trimers from aggregates (i.e., collapse aggregate into trimer), 1 ug of purified KNHI 144 SOSIP R6 trimer was incubated with Tween® 20 to a final concentration of 0.05% (v/v) at 0°C (on ice), room temperature (22-23°C) at 37°C, or left untreated for 10 minutes. Following the incubation, samples were analyzed by BN-PAGE and Coomassie staining.
Tween® 20 effect on KNHl 144 gp20: To test if Tween® 20 had a similar effect on KNHl 144 gp20, 1 ug of purified gp20 monomer was either untreated or incubated with Tween® 20 at a final concentration of 0.05% for 10 minutes at room temperature. Following the treatment, samples were analyzed by BN-PAGE and Coomassie staining.

Tween® 20 effect on α-2-macroglobulin (αM): 0.5 ug of purified α-2-macroglobulin was either untreated or treated with Tween® 20 at a final concentration of 0.05% for 10 minutes at room temperature. Reactions were analyzed via BN-PAGE, followed by Coomassie staining.

Size exclusion chromatography CSEO analysis:
All runs were performed at 4°C on the AKTA FPLC system (GE Healthcare). Each run was performed at least twice.

Molecular weight standards SEC: 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: 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.

KNHl 144 gp120 SEC analysis: 14 ug of purified KNHl 144 gp120 (either untreated or Tween® 20-treated as described above) was applied over the Superdex 200 column equilibrated in TN-500 and resolved at a flow rate of 0.4 ml/min. As a control, 10-14 ug of JR-FL gp120 was also analyzed in a similar manner.

KNHl 144 SOSIP R6 gpUO SEC analysis: 8-10 ug of purified KNHl 144 SOSIP R6 gp40 was treated with Tween® 20 at a final concentration of 0.05% for 10-30 minutes at room temperature. Treated samples were then applied over the Superdex 200 column equilibrated with TN-500 containing 0.05% Tween® 20 (TNT-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 staining. Fractions were also separated by BN-PAGE, followed by Western blot analysis with ARP 3 119 antibody.

Blue Native PAGE (BN-PAGE) and SDS-PAGE analysis:
All SDS-PAGE analysis (reduced and non-reduced) were performed using 4-12% Bis-Tris NuPage gels (Invitrogen). BN-PAGE analysis was performed as described (10). Silver stain
analysis was performed with the SilverQuest kit (Invitrogen). Coomassie G-250 stain was performed using either the SimplyBlue SafeStain or Easy-to-Use Coomassie® G-250 Stain (Invitrogen).

Antigenicity Experiments - Lectin ELISA:
Human mAbs b6 (32), bl2 (33) and 2G12 (26), HIVIg (40) 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, Austria, Vienna). For the lectin based ELISA, anti-Env antibodies 2G12, b6, bl2 and HIVIg were used. In addition, the CD4-IgG2 antibody conjugate PRO 542 (39) was also used.

ELISA plates were coated overnight at 4°C with lentil lectin powder from Lens culinaris (L9267, Sigma) at 10 µg/ml concentration. Plates were washed with PBS twice and blocked with SuperBlock (Pierce) (warmed to RT). Excess blocking agent was washed off with PBS. SEC fractions containing HMW aggregate were either untreated or treated with 0.05% Tween® 20 (v/v, final concentration) for 30 minutes at room temperature (RT) and were added at 0.3 µg/ml (diluted in PBS) and bound to the plates (via the lectin) for 4 hours at RT. Following binding, plates were washed 4 times with PBS and incubated with primary anti-Env antibodies starting at 10 µg/ml in PBS/5% milk. 4x serial dilutions were performed and incubations were performed for 3 hours at RT. Following antibody incubation, plates were washed 6 times and goat anti-human IgG (H+L) alkaline phosphatase conjugate secondary antibody (Jackson ImmunoResearch) was added at 1/4000 concentration in PBS/5% milk. Plates were washed 4 times and ELISAs were developed using the Ampak detection system (Dako Cytomation, Carpinteria, CA) as per the manufacturer's instructions.

DEAE anion exchange chromatography of Tween® 20-treated KNHI 144 SOSIP R6 epl40 trimers:
Purified KNH1144 SOSIP R6 gp140 trimers, treated either with or without 0.05% Tween® 20 (final), containing a2M contaminant in TN-75 buffer was applied over 1 ml DEAE HiTrap FF column (equilibrated in TN-75) at 0.25 ml/min at RT and flow-through (FT) fractions were collected. Following sample loading, the column was washed with TN-75 at 0.5 ml/min and wash fractions were collected. Finally, the column was eluted with TN-300 and equal amounts from each fraction were analyzed via BN-PAGE, followed by Coomassie G-250 staining.
Electron microscopy:
EM analysis of the SOSIP trimers was performed by negative stain as previously described (34, 35). Because this technique is incompatible with detergent, 20 µl of the original sample (0.5 mg/ml in TN-300) was dialyzed against BSB (0.1 M H$_3$BO$_3$, 0.025 M Na$_2$B$_4$O$_7$, 0.075 M NaCl, pH 8.3) and subsequently depleted of detergent using the Mini Detergent-OUT™ detergent removal kit (Calbiochem, La Jolla, CA) as described by the manufacturer. Two microliters of the resulting protein solution, diluted in 200 µl BSB, was affixed to carbon support membrane, stained with 1% uranyl formate, and mounted on 600 mesh copper grids for analysis. EMs were recorded at X100,000 at 100 kV on a JOEL JEM 1200 electron microscope. Measurements were made using the Image-Pro Plus software program. Fifty or more trimers were measured and analyzed statistically. The average diameter of the compact trimers formed by the SOSIP gpl40 (e.g., KNH1 144.R6 SOSIP) proteins was about 12-13 nm.

RESULTS

Expression and Purification of Trimeric KNH1 144 SOSIP R6 gpl40:

The purification of KNH1 144 SOSIP R6 gpl40 trimers typically involved three chromatography steps: GNA lectin affinity, Superdex 200 size exclusion and DEAE weak anion exchange. 53X concentrated cell culture supernatant precipitated with ammonium sulfate was clarified by centrifugation, diluted and applied over the GNA lectin affinity column to capture gpl40 proteins via (α-1, 3) mannose residues. Analysis of the ammonium sulfate precipitation using different starting concentrations of harvested cell culture supernatant (10OX to 40X) revealed that 53X was the optimum condition at which maximum α-2-macroglobulin precipitated out, with minimal envelope protein loss. While the GNA lectin column was highly efficient in capture of the gpl40 trimer, elution of the protein under even extremely mild conditions, with the competing MMP eluant, caused significant de-stabilization of the trimer and resulted in marked dissociation of the trimer into dimer and monomer species. Attempts to separate the different oligomeric gpl40 species via Superdex SEC resulted in efficient separation of the monomer from the dimer and trimer. Superdex 200 SEC of the GNA eluate yielded trimers that were free of monomers, but not of dimers. To resolve trimers away from dimers (and residually co-migrating monomers), a DEAE anion exchange step was incorporated, which led to very efficient separation of dimer from trimer, thereby yielding pure trimers at the end of the purification protocol.

SDS-PAGE analysis under reducing conditions showed that the final preparation was of high purity (at least 90%), with only the gpl20 moiety visible on the reduced gel (Figure 1, left panel, center lane). Common serum contaminants that were detectable by reducing SDS-PAGE were α-
2-macroglobulin (a2M) and BSA, which typically comprised up to -10% of the final preparation. The non-reduced gel shows intact gp40 protein on SDS-PAGE (Figure 1, left panel, right lane). In addition, little to no disulfide-linked aggregate (typically revealed as migrating much slower on a non-reducing gel) was detected. This was confirmed by anti-envelope Western blot analysis on the non-reduced gel (Figure 1, Anti-Env blot, middle panel). BN-PAGE analysis of the purified trimer revealed the purified trimer to migrate between the 669k thyroglobulin and 440k ferritin marker proteins (Figure 1, right panel, SOSIP R6). This is consistent with the migration patterns for JR-FL SOSIP gp140 which has been observed to migrate in the lower range of 669k and 440kDa (9, 10, 11). An additional slower migrating band, typically classified as high molecular weight (HMW) SOSIP aggregates and comprising about 30% of the preparation, was also detected (Figure 1, right panel, SOSIP R6, - lane). Typical HMW aggregate content ranged from 10 to 40% of the final preparation prior to non-ionic detergent treatment. Treatment of the purified preparation with Tween® 20 at a final concentration of 0.05% converted the HMW aggregate species to trimers, yielding a homogenous trimer preparation (Figure 1, right panel, SOSIP R6, + lane)(19). It should be noted that treatment with Tween® 20 also caused the treated trimer to migrate slightly more rapidly than the untreated trimer (notice faster mobility of trimer in the + lane).

Purification of the monomeric protein yielded a homogenous preparation as evident by a single band when analyzed by reducing SDS-PAGE (Figure 1, left panel, left lane) and Superdex 200 SEC. BN-PAGE analysis of the purified monomer, either in the presence or absence of Tween® 20 revealed a single migrating monomeric gp120 species, devoid of any higher order oligomers, consistent with its purity on SDS-PAGE (Figure 1, right panel, gp120/-+ lanes/.

Since Tween® 20 provided a simple and mild means to obtain homogenous trimers, further characterization of the non-ionic detergent effect was performed. A purified trimer preparation containing ~30% aggregates (e.g., monomer, dimmer and trimer) was treated with Tween® 20 at final concentrations of 0.0001% to 0.1% (v/v) (Figure 2A). The SOSIP R6 aggregates were converted to trimers at concentrations of 0.1% to 0.01% (Figure 2A, lanes 3-5). No conversion was observed at Tween® 20 concentrations of 0.001 and 0.0001% (Figure 2A, lanes 6 and 7). Close examination of the 0.01% reaction (lane 5) revealed that traces of aggregate were present, thus indicating that 0.01% Tween® 20 is probably the threshold concentration. To study the kinetics of the conversion, trimer preparations containing ~30% aggregate were incubated with Tween® 20 for 0, 5 and 10 minutes prior to analysis by BN-PAGE. As shown in Figure 2B,
both the 5 minute and 10 minute incubations completely eliminated the aggregate, indicating that the kinetics of the reaction was rapid and within about a 5 minute time span.

The effect of temperature on aggregate rearrangement was also examined. Aggregate/trimer preparations were incubated with Tween® 20 either at 0°C (on ice), room temperature (22-23°C), or 37°C. As shown in Figure 2C, conversion of aggregate to trimer occurred at all 3 temperatures, indicating that the Tween® 20 effect on aggregate was independent of temperature over this range. Similar results were obtained when Tween® 80 was used instead of Tween® 20.

Similar Tween® 20 treatment of the gpl20 monomer showed that there was no difference observed in its migratory pattern either in the presence or absence of Tween® 20, indicating that Tween® 20 did not affect the gpl20 monomer (Figure 1, right panel, gpl20, +/- lanes). In some cases, a mild increase in the staining intensity of the gpl20 monomer occurred.

To test if the detergent had a collapsive effect on another large multi-subunit protein, α2-macroglobulin (0.2 M), which is an acidic 726 kDa tetrameric glycoprotein comprised of four identical 185 kDa subunits, was incubated with Tween® 20. No change was observed in the migratory pattern of α2 M in the presence of Tween® 20, although there was a slight increase in the staining intensity of the protein.

To examine whether Tween® 20 could convert preparations containing predominantly aggregate as the major oligomeric species to resulting trimers, a KNHI 144 SOSIP R6 preparation containing >70% HMW aggregate was incubated with Tween® 20 and analyzed by BN-PAGE. As shown in Figure 2D, Tween® 20 was effective in converting the aggregate rich fraction to trimer (Figure 2D, left panel). Fractions of less purity containing HMW aggregate, dimers and monomers (Figure 2D, right panel, - lane, each species denoted by arrows), when treated with Tween® 20 also resulted in collapse of HMW aggregate to resulting trimer (Figure 2D, right panel, + lane). However, no effect on dimer or monomer migration was observed (Figure 2D, right panel, + lane, arrows), indicating that the Tween® 20 action was specific to KNHI 144 SOSIP R6 HMW aggregate and trimer. Consistent with previous observations, some increase in monomer staining was observed. Thus, these results indicate that JTvveen® 20 efficiently converts the KNHI 144 SOSIP HMW aggregate into trimeric form. According to this invention, Tween® 20 efficiently converted into trimers HMW preparations having greater than 10%, (e.g., greater than 10-40%), aggregate. Greater than 90-99%, or 100%, trimers were able to be recovered from non-ionic detergent-, e.g., Tween® 20, treated HMW aggregates.
SEC Analysis of KNH1 144 gpl20 monomer and SOSIP R6 gpl40 trimer:
Size exclusion chromatography (SEC) analysis was performed as a second means to characterize the molecular sizes of KNH1 144 gpl20 monomer and SOSIP R6 gpl40 trimer proteins. A Superdex 200 size exclusion column was calibrated with thyroglobulin (669 kDa), ferritin (440 kDa), BSA (67 kDa) and RNAse A (13.7 kDa) as molecular weight standards. In addition, monomeric JR-FL gpl20 was also analyzed as a control. KNH1 144 gpl20 and JR-FL gpl20 were each found to migrate at an apparent molecular weight of 210 kDa. These values are consistent with those found for JR-FL gp120 (10).

To further study the oligomeric nature of the KNH1 144 SOSIP R6 gpl40 trimer, final purified preparations were treated with Tween® 20 prior to analysis on Superdex 200 SEC to yield homogenous and unambiguous trimer samples devoid of HMW aggregate. Initial studies showed re-formation of HMW aggregate when treated trimer samples were resolved in non-detergent TN-500 buffer on the SEC column. The resulting mixed trimer-aggregate fractions, presumably re-formed upon separation of the Tween® 20 from the gpl40 oligomers in non-detergent buffer, was considered unsuitable for SEC analysis due to its heterogeneous nature.

In order to maintain homogenous (rimers, treated trimer was resolved in the presence of TN-500 containing 0.05% Tween® 20 (TNT-500). As shown in Figure 3, the trimer (thick arrow) migrated from fractions BIO through C2, represented in the major peak, with its peak signal at fraction B12 (vertical arrow). The retention time at this fraction corresponds to an apparent calculated molecular weight of ~518 kDa. The reported apparent molecular weight (MW) of JR-FL SOSIP gpl40 trimer calculated via Superdex 200 SEC analysis is ~520 kDa (9); and thus, the calculated apparent MW value for KNH1 144 SOSIP R6 gpl40 trimer is consistent with MW values of other SOSIP envelope trimers.

Effect of Tween® 20 Treatment on KNH1 144 SOSIP R6 Antigenicity:
Studies of the antigenic properties of unpurified KNH1 144 SOSIP R6 gpl40 (19) showed that it was immunoprecipitated by the neutralizing molecules 2G12, bl2, CD4-IgG2, as well as the non-neutralizing mAb b6. The experiments described herein further assessed the effect of the Tween® 20 aggregate collapse on the antigenic properties of KNH1 144 SOSIP HMW aggregates to determine if conversion of HMW aggregate into trimer favorably enhanced antigenicity.
SEC fractions containing ≥ 80% KNHl 144 SOSIP R6 HMW aggregate content (as shown in Figure 2D, lane) were either untreated or Tween® 20 treated (typical reaction is represented in Figure 2D). The antigenicity of the proteins in the presence and absence of Tween® 20 was examined using a lectin based ELISA. These results are shown in Figure 3A. The anti-env antibodies and CD4-IgG2, displayed increased binding to the Tween® 20 treated aggregate. The above experiments were performed on Tween® 20 converted trimer, using preps containing >80% HMW aggregate.

To demonstrate that Tween® 20 treatment did not unfavorably disrupt the above antibody epitopes on trimers, similar lectin ELISAs were performed using 2G12, b6, b12 and CD4-ιgG2 on SOSIP R6 gpl40 trimers that contained low amounts of HMW aggregate (~10-15% content) that were either untreated or treated with Tween® 20. As shown in Figure 3B, no significant differences were observed in the antigenicity of trimer in presence or absence of Tween® 20. Unfortunately, since the HMW aggregate species is present in very limiting quantities, the Tween® 20 effect was assessed using only the above mentioned mAbs. These results show that Tween® 20 treatment and consequential conversion of HMW aggregate to resulting trimer enhances epitope exposure for Env binding antibodies. Thus Tween® 20 treatment and presence may offer favorable consequences in the context of KNHl 144 SOSIP R6 gpl40 trimer stability and antibody epitope exposure.

Effect of Tween® 20 Treatment on the Ionic Properties of KNHl 144 SOSIP R6 gpl40 trimer: DEAE anion exchange chromatography was used to examine the effect of Tween® 20 on the ionic properties of SOSIP R6 gpl40 and control proteins. Untreated or Tween® 20 treated KNHl 144 SOSIP R6 gpl40 trimer spiked with a2M containing protein (which is unaffected by Tween® 20 and binds to anion exchange resins) were applied over DEAE anion exchange column (Figure 4, Load). The column was washed and eluted and fractions were analyzed via BN-PAGE and Coomassie staining and is shown in Figure 4. As expected, untreated SOSIP R6 gpl40 trimer and the a2M contaminant bound to the DEAE column and were recovered in the elution fraction (Figure 4, Untreated control, top panel, denoted by asterisks). However, upon treatment with Tween® 20, the KNH1144 SOSIP R6 gpl40 trimer was found in the flow-through (FT) fractions of the column (Figure 4, Tween® 20 treated, bottom panel, FT, denoted by asterisks), indicating that it did not bind to the DEAE, unlike the untreated trimer. Residual trimer is further recovered in the wash fraction (Figure 4, Wash). In contrast, the a2M contaminant, which was used as the internal control, bound to the DEAE column and was
recovered in the elution, indicating that it was unaffected by the presence of Tween® 20 (Figure I, Tween® 20 treated, bottom panel, Elution).

In other similar experiments, in which BSA, another acidic protein was substituted as the contaminant, similar results were obtained. This indicates that Tween® 20 treatment may exert its action on KNH144 SOSIP R6 HMW aggregate and trimer through a combination of hydrophobic interactions that possibly involve perturbations in inter- and/or intra-subunit charge-charge interactions, as examined by DEAE anion exchange chromatography.

Electron Microscopy and Digital Imaging of KNH 144 SOSIP R6 gpl40 trimers: Electron microscopy was performed on purified SOSIP R6 preparations employing negative stain EM analysis. The results, shown in Figure 6, reveal that the majority of the observed structures displayed a regular compact morphology with approximate three-fold symmetry. This tri-lobed configuration is most apparent in preparations with deeper stain (Figure 6; panel of trimers) that are less subject to the flattening that can occur in thinner staining preparations.

Initially, for the EM studies, it was found that the uranyl formate negative straining technique was not compatible with detergent-containing buffers. However, some trimeric structures of the anticipated dimensions were observed in the poorly staining preparations. Thereafter, the KNH 144 SOSIP preparation was subjected to a detergent removal protocol, which yielded improved staining. Following detergent removal, the majority of the observed structures displayed a regular compact morphology with approximate three-fold symmetry (e.g., Fig. 6). This configuration is most apparent in preparations with deeper stain (Fig. 6B) that are less subject to the flattening that can occur in thinner staining preparations (Fig 6A).

In order to calculate diameters of the trimers, 70 spikes in the shallow stain samples were scored and a diameter of 13.5 ± 1.73 nm was calculated. Seventy eight (78) trimers from the deep stain were scored and resulted in a diameter of about 11.6 nm ± 1.75 nm. The shallow stain preparation likely gives a slight overestimation of the size and the deep stain preparation gives a slightly underestimated size. Therefore, the true size is likely to be about 12.6 ± 1.74 nm and in line with authentic Env spikes measured in situ on both negatively stained, as well as unstained, cryo-EM preparations of SIV (36, 37). Thus the biophysical EM analysis of KNH 144 SOSIP R6 gpl40 is in good agreement with the above biochemical data and confirms the oligomeric status of the purified KNH 144 env complex as being trimeric.
DISCUSSION
In the context of identifying and pursuing a variety of HIV-I Env-based protein vaccines, described herein is the purification and characterization of a novel subtype A KNHl 144 trimeric envelope spike protein and its properties. Several novel insights were gained as a result of these studies, which revealed the biochemical effects of Tween® 20 on the oligomeric conformations of the KNHl 144 SOSIP R6 proteins. Until the present invention, only one subtype B envelope, HIV-I JR-FL has been manipulated to a purified form to mimic as closely as possible the native trimeric structure of the HIV-I viral surface envelope complex via the SOSIP technology (8-11, 15-17). The present invention provides another clade, clade A KNHl 144, for which the SOSIP technology results in purified trimeric envelopes that are stable, soluble, and fully cleaved.

The purification process implemented according to the present invention for the KNHl 144 SOSIP trimers provides a marked improvement over that utilized for JR-FL SOSIP gp140 trimers. For the KNHl 144 SOSIP1, the GNA lectin column provided a significant enrichment of gp140 proteins, but elution off the column significantly destabilized the gp140 trimers, resulting in a compromise of trimer fidelity on the column. As a result, significant dissociation of the trimer to resulting dimer and monomer was noticed. This destabilization could be brought about from Galanthus Nivalis lectin binding to αl-3 and αl-6 mannose linkages on the gp140 high mannose chains, which are internal linkages and not terminal linkages (20). During elution, the affinity of the lectin for the mannan is likely much higher than the intersubunit protein-protein affinities of the 3 gp120-gp41 C E monomers contributing to trimer formation, resulting in destabilization and dissociation into component dimers and monomers. To alleviate some measure of the destabilization that could be caused due to resulting shear stresses during elution, a one hour incubation in MMP eluting buffer was included. So while a highly enriching step, the lectin affinity column also decreased the final yield of trimer significantly, due to its dissociation during the elution phase.

The next step in the purification, Superdex 200 SEC, while somewhat efficient in resolving away monomer, was not very effective in resolution of dimer from trimer. The incorporation of a DEAE weak anion exchange chromatography step was very efficient in resolving dimer (and residual monomer) away from trimer, resulting in trimeric KNHl 144 SOSIP R6 gp140 of high purity. Notably, binding (and retention) of the trimer occurred under a relatively polar environment (ys-α-ys ion exchange) at 75 mM NaCl, while dimer and monomer flowed through the DEAE column under these conditions.
It is relevant to extrapolate from its behavior on anion exchange chromatography that the nature of the KNH! 144 SOSIP R6 gpl40 trimer is that of an acidic protein, which would be contrary to its predicted basic isoelectric point (pi) of 8.73 calculated for the protein backbone. However, the likely presence of the predicted acidic sialylated complex oligosaccharide chains on the gpl40 (21, 22) would contribute to a decrease in the overall charge of the glycoprotein and thus confer on it properties of an acidic protein. Indeed, analysis of purified KNH! 144 SOSIP R6 gpl40 trimers on isoelectric focusing gels reveal it to migrate at a pi range of 5.9 to 6.1, consistent with the above observations.

The purified trimer was shown to contain variable amounts of HMW aggregate (Figure 1, right panel, BN-PAGE), which could not be attributed to being formed at any one particular step of the purification, although one possibility might be at the lectin elution step. As mentioned before, one of the key improvements made in this purification protocol is absence of SDS-insoluble aggregates in the final prep, which are formed by abberantly formed disulfide bonds and are visualized by their slow migration on a non-reduced SDS-PAGE. As detected by Coomassie staining and confirmed by anti-envelope Western blot, little to no SDS-insoluble aggregates were observed (Figure 1, left and middle panels, Non-Red SDS-PAGE and Anti-Env blot). This is in contrast to what was observed with JR-FL SOSIP gpl40 (R6 and non-R6 versions), where SDS-insoluble aggregates comprised a significant percentage of the final preparations (9, 10, 11).

Based on observations regarding non-ionic detergent treatments of KNH! 144 SOSIP R6 gpl40 trimers (19), Tween® 20 was used to address the co-purifying HMW aggregate present in the final trimer preparations. Tween® 20 was chosen because initial observations had shown that Tween® 20 treatment was mild and did not result in any detectable monomer formation, unlike treatment with the other non-ionic detergents NP-40 and Triton X-1OO, where diners and monomers were observed upon treatment (19). Tween® 20 treatment of the final purified KNH! 144 SOSIP R6 trimer preparation was highly reproducible and resulted in the "conversion" of the HMW aggregate species, as shown in Figure 1 (right panel, BN-PAGE). Since this resulted in a single, homogenous, oligomeric species of KNH! 144 SOSIP R6 gpl40 trimers, we routinely incorporated it as the final step in our preparations. Further analysis using reduced SDS-PAGE gels showed that the purified trimer was fully cleaved, with practically undetectable uncleaved protein (as visualized by both Coomassie staining and Western blot analysis) (Figure 1, left panel. Red SDS-PAGE). The initial purifications were performed using a non-R6 version of KNH! 144 SOSIP gpl40, which resulted in 40-50% of uncleaved protein in the final preparation, prompting the development of the R6 version. This also represents another
improvement over JR-FL SOSIP R6 gpl40 trimers, where cleavage of gp120-gp41Ec to was not as efficient (9, 11).

In order to expand the initial Tween® 20 observations to the stability of HMW aggregates, a variety of experiments were performed to characterize the effect of Tween® 20 and to better understand its mechanism of action. As shown in Figure 6, the effect of Tween® 20 is dose dependent, time dependent and temperature independent within the parameters that were examined. Its effect is remarkably specific to KNHI 144 SOSIP R6 HMW aggregate and trimers and has no effect on gp20 monomers, or KNHI 144 SOSIP R6 dimers. In addition, other similar large, macromolecular, acidic proteins such as α2M are not affected by the detergent. Initially, the hypothesis was that the Tween® 20 specifically interacted with points of gp120-gp41 Ecto intersubunit contact within the HMW aggregate, presumably in a hydrophobic manner. In this context, the HMW aggregate would have to be comprised of some multiple of trimer (most likely a dimer of trimers), since detergent treatment specifically results in a "rearrangement" to a trimeric configuration. The specificity of this reaction can further be defined by the observation that dimeric KNHI 144 SOSIP R6 gpl40 proteins are unaffected and do not undergo the collapse (Figure 6D). In addition, Tween® 20 treatment would also seem to cause the trimer to assume a more compact configuration, as evident by its slightly more rapid mobility on BN-PAGE (Figure 1).

While the anti-flocculatory effects of non-ionic detergents on aggregates of macromolecular proteins such as antibodies (immunoglobulins, for example) are well known and documented, the mechanisms of their actions have been realized to be largely by pre-emption of unfavorable hydrophobic interactions by detergent intercalation. Tween® 20, however, would seem to exert its action in a somewhat paradoxical mechanism, since treatment of the KNHI 144 SOSIP R6 gpl40 trimer with the detergent renders it unable to interact with anion exchange resins such as DEAE (Figure 1, bottom panel, Tween® 20 treated), indicating that the overall charge of the trimer was being affected by the detergent.

Since the nature of non-ionic detergents is exactly that, i.e., non-ionic, it is difficult to realize how an uncharged molecule such as Tween® 20 would affect the charge status of a large, macromolecular oligomer such as the KNHI 144 SOSIP R6 trimer. Furthermore, this effect is highly specific to the trimer, as other such large, highly charged (acidic) oligomeric proteins such as α2M and even smaller ones such as BSA are unaffected by the detergent. One hypothesis that has emerged from this invention is that perhaps the Tween® 20 was "coating" the trimer in a
manner that may cause perturbations in its conformation, resulting in its "compactness". These perturbations would be of a subtle nature which involve the various points of contact between the individual component gp140 monomers, causing disruption and destabilization of interactions that favor the HMW aggregate conformation. A consequence of these perturbations would be "shielding" of ionic charges that would normally be exposed (and contribute to binding to ion exchange resins). It is reasonable to speculate that perhaps the charges that are "shielded" are those on the sialic acid residues of the complex carbohydrate chains, since these would be most likely to be highly exposed at the surface (21, 22). Tween® 20 and Tween® 80 are polyoxethylene sorbitan esters of fatty acids and thus may likely interact with the sialic acids, causing a charge "neutralization" effect. The involvement of the sialic acid residues can be investigated by mild sialyrase treatment (21, 22) and removal of these residues, followed by Tween® 20 treatment, followed by monitoring of binding on ion exchange resins.

To further biochemically characterize the purified KNH1144 monomeric and trimeric envelope proteins, size exclusion chromatography analyses were performed in order to ascertain their apparent molecular masses. These were performed on Tween® 20 treated trimers that were devoid of any HMW aggregates and thus consisted of only one homogeneously oligomeric species, i.e., the trimer, and therefore would yield unambiguous results. The retention times of the KNH1144 SOSIP R6 gp140 trimer resulted in a calculated apparent molecular weight of 518 kDa. This is consistent with the reported calculated apparent molecular weight of 520 kDa for the other SOSIP gp140 trimer, JR-FL SOSIP gp140 (9). The predicted molecular weight for a trimer such as KNH1144 (and JR-FL) would be 420 kDa (3 x 140 kDa monomers). Thus, similar to JR-FL SOSIP gp140, the KNH1144 SOSIP R6 gp140 trimer also exhibits an abberant migration on SEC, presumably due to interactions of its N-linked glycans with the dextran-(agarose polymer) based matrix of Superdex 200, resulting in a higher than expected apparent molecular mass. In addition, envelope proteins have been shown to be non-globular in shape (10, 23, 24); therefore, gel filtration may not be optimal for determination of their precise molecular masses. This also extends to the KNH1144 gp120 monomer as well. Values of —210 kDa were obtained for KNH1144 gp120 and the control JR-FL gp120. The reported value for JR-FL gp120 is 200 kDa (10); accordingly, the obtained values are well within the expected range (given that molecular weight determination via SEC is not extremely accurate, unlike other methodologies such as mass spectrometry). Thus, gp120, whose predicted molecular weight ranges from ~95 to —120 kDa, results in an abberant migratory pattern on SEC, presumably due to its glycan interactions with the sizing column matrix. It should be noted that unlike the KNH1144 SOSIP R6 gp40 trimer, migration of KNH1144 gp120 (and JR-FL gp120) were not
affected by the presence or absence of Tween® 20, consistent with the initial BN-PAGE observations (Figure 1, right panel, gpl20).

While it would seem that the presence of Tween® 20 for KNHI 144 SOSIP R6 gpl40 proteins would be advantageous, possible Tween® 20 effects on the antigenicity of the HMW aggregate and trimer were examined. Effects on antigenicity was examined by performing lectin ELISAs with the NAbs 2G12, b12, HIVIg, the CD4-IgG2 antibody conjugate PRO 542, as well as the non-neutralizing mAb b6, to gain information on neutralizing/non-neutralizing epitope exposure and accessibility. It was reasoned that trimer preparations containing 10-30% HMW aggregate may not undergo significant enough changes that would be detectable in a non-quantitative assay such as IPs, i.e., subtle changes (20-30% changes) may go undetected in such an assay due to sensitivity. However, samples representing extremes may undergo significantly high changes that should be detectable in an assay format such as ELISA. Therefore, SEC fractions that contained ≥ 80% HMW aggregate were used, which would reflect one extreme prior to Tween® 20 treatment and the resulting trimer, which would reflect the other extreme post treatment. A representative reaction of this is illustrated in Figure 2D.

As shown in Figure 4A, significant epitope exposures were observed upon Tween® 20 rearrangement of the HMW aggregate to trimer, and these changes were noticed for all of the anti-env agents. These changes indeed were not as apparent in trimer preparations that were predominantly trimer, with low aggregate content (10-15%) (Figure 4B). Thus the treated, purified trimer displays antigenic properties similar to that which was previously observed with crude, unpurified trimer supernatants, i.e., binding to 2G12, b6, b!2 and PRO 542 (19). In the context of HIVIg, which is a low neutralizing polyclonal human antisera directed against gpl20 hypervariable loop (40), it can be inferred that this epitope is accessible on the surface of the HMW aggregate, based on its ability to bind the antibody in absence of Tween® 20. Consistent with the other anti-Env agents examined here, HIVIg epitope exposure also significantly increased on the rearranged trimer, upon treatment with Tween® 20. The likely explanation to these increases in epitope exposure is that "disruption/rearrangement" of the aggregate and its subsequent conversion to trimer unshields the above mentioned surfaces and thus, upon conversion, these surfaces are now exposed on their individual trimers and are accessible to the antibodies. From the context of a single HMW aggregate which is likely to be a multimer of trimers, only a small portion of these epitopes are accessible, most probably due to steric hindrance from adjacently "clumped" SOSIP R6 trimers/oligomers. When the single HMW aggregate is then Tween® 20 converted to resulting trimers, antibody epitopes are now exposed
on every one of the resulting individual component trimers, resulting in an increase in antibody accessibility and binding. Thus Tween® 20 treatment and its conversion of the aggregate to trimer do not seem to have detrimental effects on antigenicity and may be favorable to the structural properties of the KNHI 144 SOSIP R6 gpl40 proteins.

Analysis of KNHI 144 SOSIP R6 gpl40 proteins by negative stain EM further confirmed the biochemical observations that these gpl40 proteins were indeed trimeric in nature (Figure 6). A distinguishing feature of the KNHI 144 SOSIP R6 construct, in comparison to other similar constructs of trimerized gpl20 and gpl40, is its compact nature. Most other constructs show either predominantly loosely associated subunits or a mix of loosely and tightly associated subunits (5, 18, 38). The observation that the KNHI 144 SOSIP R6 trimer is compact is associated with anti-Env antibody epitope availability. EM on Tween®-treated trimer which has favorable anti-Env epitope exposure was performed. It is somewhat incongruous from a purely steric standpoint that a "compact" trimer would also have improved epitope exposure, a consequence expected from a "loose" or "elongated" structure. Immunoelectron microscopy analyses with the above mentioned antibodies will further address the exposure of epitopes on trimeric forms.

The present invention expands the panel of trimeric HTV-I envelope proteins that may be used as protein-based HIV-I vaccine candidates or serve as a template for future design of Env based protein vaccine candidates, using the SOSIP technology. Immunological studies in rabbits with JR-FL SOSIP R6 gpl40 trimers, while effective in eliciting NAbs, were limited in their breadth of neutralization of primary HIV-I isolates (11). Factors associated with the biochemical nature of the JR-FL SOSIP gpl40 and other oligomeric Env proteins that are thought to limit their observed immunological response in animals, such as inefficient furin cleavage of the gpl20-gp41EcN cleavage site giving rise to heterogenous trimers (containing both cleaved and uncleaved trimers), presence of SDS-insoluble aggregates and presence of undesirable gpl40 oligomers such as dimers and monomers (5, 6, 9, 10, 11, 27-30) have been issues needing resolution.

The description of the KNHI 144 SOSIP R6 gpl40 trimers of the present invention addresses most of these issues. Furthermore, the description of the Tween® 20 affects on converting HMW aggregates to trimeric forms further expands on current knowledge of the aggregate species in HIV-I biology. Of significance, it was shown for the first time, that oligomeric Env protein complexes designed using the SOSIP technology platform are indeed trimeric from EM images
and that the trimers are of a similar diameter as native spikes on the HIV-I virion (36, 37). Expansion of the panel of potential HIV-I SOSIP protein vaccine candidates by development of a clade A envelope according to this invention now allows for immunological evaluation of the KNHI 144 SOSIP R6 gpl40 trimer in small animals, for example. Such evaluations will assist in determining the efficacy of KNHI 144 SOSIP R6 gpl40 trimers as immunogens capable of eliciting broadly neutralizing immune responses directed against HIV-I.

REFERENCES
EXPERIMENTAL DETAILS

MATERIALS AND METHODS

Subtype B 5768.4 SOSIP R6 gp140 expression and purification:
The subtype B 5768.4 envelope sequence has been described (8). The sequence was modified to
make the soluble SOSIP R6 gp140 version, as described above for the KNH1 144 isolate in the
section "Experimental Details I" and for the JR-FL SOSIP R6 gp140 trimers (3, 4). DNA
synthesis was performed by DNA 2.0 (Menlo Park, CA). The 5768.4 SOSIP R6 gp140 trimer
was expressed in HEK293 and small scale (2 L) purification was performed as described above
for KNH1 144 SOSIP R6 gp140.

Detergent Aggregate "Collapse"/"Conversion" Experiments:
Tween® 20 Dose effect: 1 ug of purified 5768.4 SOSIP R6 trimer was incubated with varying
concentrations of Tween® 20 ranging from 0.1 to 0.0001% (v/v) and incubated for 1 hour at
room temperature. Following incubation, samples were analyzed by BN-PAGE as described
above.

Detergent effect on 5768.4 SOSIP R6 gp140 trimer preparations: 0.24 ug of purified 5768.4
SOSIP R6 trimer was incubated with Triton X-100, NP-40, or SDS to a final detergent
concentration of 0.1% (v/v) or with Tween® 20 at concentrations of 0.05 and 0.1% for 1 hour at
room temperature. Following incubation, 4x BN-PAGE MOPS sample buffer was added and the
samples were immediately analyzed on BN-PAGE at 150 V for 2 hours at room temperature,
followed by Coomassie G-250 staining.

Size exclusion chromatography (SEO analysis):
All runs were performed at 4°C on the AKTA FPLC system (GE Healthcare). Each run was
performed at least twice.

Molecular weight standards SEC: 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: thyroglobulin 669,000 Da; ferritin 440,000 Da; BSA 67,000 Da; 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.
Blue Native PAGE CBN-PAGE, SDS-PAGE and Western blot analysis:

A SDS-PAGE analysis (reduced and non-reduced) were performed using 4-12% Bis-Tris NuPage gels (Invitrogen). BN-PAGE analysis was performed as described before (1-3). Silver staining analysis was performed with the SilverQuest kit (Invitrogen).

RESULTS AND DISCUSSION

Detergent "collapse" effect on subtype B 5768.4 HMW aggregate:

Detergent treatments were performed on a trimeric gpl40 of a different subtype 5768.4, which is a subtype B envelope (8). The 5768.4 Env protein was modified to the SOSIP R6 version, expressed and purified as a gpl40 trimer. The purified final preparation contained high HMW aggregate content (~60%) and minor α2M contamination (~5%), with trimer comprising the rest. Purified preparations were incubated with the various indicated detergents (Triton X-100, NP40, SDS, or Tween® 20) and were treated to collapse HWM aggregate.

Tween® (Tween® 20 and Tween® 80) effectively collapsed HMW aggregate to trimers at 0.1 and 0.05% concentrations. Triton X-100 was also capable of collapsing HMW aggregate to trimer, however, some breakdown to monomeric 5768.4 was observed. As expected, SDS was effective in breaking down the entire 5768.4 gpl40 protein to resulting monomers by virtue of its denaturing effect on the trimer and HMW aggregate. NP40 treatment also led to collapse of HMW aggregate to trimer, but the resulting trimer displayed a somewhat broader staining compared with that of Tween® 20 treated trimers. Thus, the detergent effect, in particular, Tween® 20, on the HMW aggregate is not unique to KNH1144 SOSIP gpl40 Env proteins and exhibits similar HMW aggregate collapse ability on other subtypes of HIV envelope trimers as well, such as the 5768.4 SOSIP R6 gpl40.

REFERENCES FOR EXPERIMENTAL DETAILS II


**EXPERIMENTAL DETAILS in**

Purification of SOSIP Env Trimers:
An efficient purification process was developed to purify SOSIP Env trimers and to increase the yield of trimers. This process is exemplified by using a preparation, e.g., a cell culture fluid containing KNH1 144.R6 SOSIP proteins, to purify KNH1 144.R6 SOSIP trimers.

In this process, concentrated cell culture fluid (CCF) preparation containing KNH1 144 SOSIP.R6 gpl40 trimers, dimers and monomers was subjected to ammonium sulfate precipitation at 4°C to remove contaminant proteins, e.g. macroglobulin. The resulting supernatant was applied to a lectin (Galanthus Nivalis Lectin (GNL) Vector Laboratories Burlingame, CA) chromatography column (e.g., GEHC (GE Healthcare XK), at 4°C. A linear gradient elution was used and bound proteins were eluted to PBS, pH 7.25, 1.0 M methyl-a-D-mannopyranoside (MMP) in ten column volumes (CV) followed by 5 CV at 100% PBS, pH 7.25 +1.0 M MMP. Fraction size was 0.5 CV and about 30 fractions were collected. Macroglobulin and monomer eluted first and were essentially removed from the trimer product. Column fractions were analyzed by gel electrophoresis (BN-PAGE) and the fractions enriched in trimer were pooled based on the gel analysis.

The trimer-containing eluted fractions from the lectin chromatography column were applied to a first DEAE sepharose column (DEAE 1) in the absence of Tween 20®. A 5-ml HiTrap DEAE FF sepharose column (GE Healthcare/Amersham Biosciences Piscataway, NJ) was used in this step. The DEAE 1 chromatography was performed at room temperature. The column equilibration buffer comprised 20 mM Tris, 0.075 M NaCl, pH 8.0, and the column equilibration flow rate was 10 ml/min. The column loading and elution flow rate was 2.5 ml/min with a fraction size of 2.5 ml. The high flow rate allowed this step to be completed in a short amount of time. The KNH1 144.R6 gpHO trimer product was eluted from DEAE 1 in several fractions through a linear gradient to 20 mM Tris, 0.3 M NaCl, pH 8.0 in ten column volumes (10 CV). KNH1 144 SOSIP.R6 gpl40 monomers and dimers were removed in the flow through and wash step using 20 mM Tris, 75 mM NaCl (pH 7.5) at room temperature.
A second Hi-Trap DEAE FF sepharose column (DEAE 2) was equilibrated with buffer containing Tween 20® (20 mM Tris, 75 mM NaCl, 0.05% Tween, pH 8.0). The DEAE 2 chromatography was also performed at room temperature. The DEAE 1 elution fractions containing KNHI 144 SOSIP.R6 trimers in buffer also containing Tween 20® was applied to the second DEAE column. The KNHI 144 SOSIP.R6 trimer product was obtained in the flow-through and in the wash pool, since KNHI 144 SOSIP.R6 trimer did not bind to the DEAE column in the presence of Tween 20® in the buffer. A suitable range of Tween 20® for purification of the SOSIP.R6 trimers is 0.025% to 1%. 0.05% Tween 20® was used in many purification runs.

In this example, a high quality KNH 1144 SOSIP.R6 trimer product was purified as shown in Figures HA and HB. Only a single band was observed in the BN-PAGE analysis (Fig. 11A). SDS-PAGE analysis demonstrated that there was no uncleaved gp140 in the product (Fig. 11B). A high purity trimer product was obtained using the above-described process compared with other purification methods, as shown in Figures 12A and 12B. The immunogenicity of the KNHI 144 SOSIP.R6 trimer product was also tested by immunoprecipitation (IP) experiments, e.g., as shown in Figure 13 using ARP 3119 probing antibody (also known as CA13) (MRC Centralized Facility for AIDS reagents, NIBSC, UK). 2G12, b12, b6, and 15e are HTV neutralizing antibodies used in the IP experiment. PRO542 is a CD4-IgG2 heterotetrameric protein. 75 ug KNHI 144 SOSIP.R6 trimer was purified using this purification method from 1 liter (IL) of cell culture fluid (CCF). At least 50ug of trimer product was recovered from IL of CCF using the purification method described in this example. The resulting purified and enriched trimer product was free from aggregates, monomers and dimers. Monomer content was less than 5% based on BN-PAGE/silver stain gel analysis.
What is claimed is:

1. A process of recovering trimers of a retroviral envelope (Env) protein from a preparation comprising trimers of the retroviral Env protein and high molecular weight aggregates thereof which comprises treating the preparation with a non-ionic detergent so as to thereby convert the high molecular weight aggregates into trimers, and recovering the trimers from the preparation so treated.

2. The process of claim 1, wherein the retroviral Env protein is an HTV Env protein.

3. The process of claim 2, wherein the retroviral Env protein is an HIV-I or HIV-2 Env protein.

4. The process of claim 1, further comprising formulating the recovered trimers with a pharmaceutically acceptable carrier, excipient, or diluent.

5. A process of recovering trimers of a retroviral envelope (Env) protein from a preparation comprising trimers of the Env protein and high molecular weight aggregates thereof which comprises:
   (a) subjecting the preparation to lectin chromatography to obtain a trimer-enriched eluate in which aggregate content is reduced;
   (b) applying the eluate of step (a) to a first diethylaminoethyl (DEAE) sepharose chromatography column in the absence of non-ionic detergent to obtain elution fractions further enriched in trimers;
   (c) applying trimer-enriched elution fractions from the first DEAE sepharose chromatography column of step (b) to a second DEAE sepharose chromatography column in the presence of non-ionic detergent; and
   (d) collecting the flow through and wash fractions from the second DEAE sepharose chromatography column in the presence of non-ionic detergent, so as to thereby recover the trimers of the retroviral Env protein.

6. The process of claim 5, wherein the retroviral Env protein is an HW Env protein.

7. The process of claim 6, wherein the retroviral Env protein is an HIV-I or HIV-2 Env protein.
8. The process of claim 5, wherein, in step (a), the preparation is a concentrated cell culture fluid.

9. The process of claim 5, wherein, in step (a), the preparation is subjected to ammonium sulfate precipitation prior to the lectin chromatography.

10. The process of claim 5, wherein, in step (a), column fractions enriched in trimers are eluted through a linear gradient in PBS to 1.0 M methyl-a-D-mannopyranoside (MMP) in ten column volumes.

11. The process of claim 5, wherein, in step (b), fractions further enriched in trimers are eluted from the DEAE chromatography column through a linear gradient to 20 mM Tris, 0.3 M NaCl, pH 8.0 in ten column volumes.

12. The process of claim 5, wherein, in step (c), the fractions further enriched in trimers are eluted from the second DEAE chromatography column through a linear gradient to 20 mM Tris, 0.3 M NaCl, pH 8.0 in ten column volumes.

13. The process of claim 5, wherein the wash fractions of step (d) comprise buffer which comprises 20 mM Tris and 75 mM NaCl, pH 7.5.

14. A process of recovering trimers of a retroviral envelope (Env) protein from a preparation comprising trimers of the retroviral Env protein and high molecular weight aggregates thereof, which comprises:

(a) subjecting the preparation to lectin affinity chromatography to obtain an eluate enriched in trimers and essentially separated from monomer and dimer aggregates and macroglobulin; and

(b) subjecting the eluate of step (a) to a first diethylaminoethyl sepharose chromatography column in the absence of non-ionic detergent so as to further remove aggregates in the flow through; and

(c) subjecting the eluate of step (b) to a second diethylaminoethyl sepharose chromatography column in the presence of non-ionic detergent so as to recover the trimers of the retroviral Env protein.
15. The process of claim 14, wherein the retroviral Env protein is an HIV Env protein.

16. The process of claim 15, wherein the retroviral Env protein is an HIV-I or HIV-2 Env protein.

17. The process of claim 14, wherein, in step (a), the preparation is a concentrated cell culture fluid.

18. The process of claim 14, wherein, in step (a), the preparation is subjected to ammonium sulfate precipitation prior to the lectin chromatography.

19. The process of claim 1, claim 5, or claim 14, wherein the trimers are recovered in the presence of the non-ionic detergent so as to maintain trimer stability upon subsequent storage.

20. The process of claim 1, claim 5, or claim 14, wherein the non-ionic detergent is a polyethylene type detergent.

21. The process of claim 20, wherein the polyethylene type detergent is a poly (oxyethylene) sorbitan monolaureate.

22. The process of claim 21, wherein the poly (oxyethylene) sorbitan monolaureate is poly (oxyethylene) (20) sorbitan monolaureate.

23. The process of claim 20, wherein the polyethylene type detergent is poly (oxyethylene) sorbitan monooleate.

24. The process of claim 1, claim 5, or claim 14, wherein the Env protein comprises consecutive amino acids having a sequence corresponding to that of a naturally occurring Env protein of an HFV-I subtype A isolate or a mutant derived therefrom.

25. The process of claim 24, wherein the HIV-I subtype A isolate is KNH1 144.
26. The process of claim 1, claim 5, or claim 14, wherein the Env protein comprises consecutive amino acids having a sequence corresponding to that of a naturally occurring Env protein of an HIV-I subtype B isolate or a mutant derived therefrom.

27. The process of claim 26, wherein the HIV-I subtype B isolate is 5768.4.

28. The process of claim 5, wherein steps (a) through (d) are carried out at room temperature.

29. The process of claim 14, wherein steps (a) through (c) are carried out at room temperature.

30. The process of any of claims 1, 5, or 14, wherein the non-ionic detergent present is at a concentration of 0.001% to 1% by weight of the preparation.

31. The process of claim 30, wherein the concentration of the non-ionic detergent is from 0.01% to 1% by weight.

32. The process of claim 31, wherein the concentration of the non-ionic detergent is from 0.025% to 1% by weight.

33. The process of claim 31, wherein the concentration of the non-ionic detergent is from 0.01% to 0.5% by weight.

34. The process of claim 33, wherein the concentration of the non-ionic detergent is from 0.01% to 0.1% by weight.

35. The process of claim 34, wherein the concentration of the non-ionic detergent is from 0.01% to 0.05% by weight.

36. The process of claim 35, wherein the concentration of the non-ionic detergent is 0.05% by weight.

37. The process of any of claims 1, 5, or 14, wherein prior to treatment with the non-ionic detergent the preparation comprises greater than 10% high molecular weight aggregates.
38. The process of claim 37, wherein prior to treatment with the non-ionic detergent the preparation comprises 10-40% high molecular weight aggregates.

39. The process of any of claims 1, 5, or 14, wherein prior to treatment with the non-ionic detergent the preparation comprises greater than 70% high molecular weight aggregates.

40. The process of any of claims 1, 5, or 14, wherein the Env protein comprises (a) a first polypeptide which comprises consecutive amino acids, the sequence of which corresponds to the sequence of a modified gpl20 envelope polypeptide portion of a gpl40 envelope of an HTV-I KNHI 144 isolate, or a quasi-species thereof; and (b) a second polypeptide which comprises consecutive amino acids, the sequence of which corresponds to the sequence of a modified gp41 ectodomain polypeptide portion of the gpl40 envelope of the HIV-I KNHI 144 isolate or such quasi-species thereof, the sequence of said modified gpl20 envelope polypeptide portion and said modified gp41 ectodomain polypeptide portion of said HIV-I KNHI 144 isolate being as set forth in SEQ ID NO:2 and SEQ ID NO:3, respectively, said modified gpl20 envelope polypeptide portion comprising a cysteine at amino acid position 511 and said modified gp41 ectodomain polypeptide portion comprising a cysteine at amino acid position 617 and a proline at amino acid position 571, wherein (i) the amino acid positions are numbered by reference to SEQ ID NO:1, (ii) the modified gpl20 envelope polypeptide portion further comprises a mutated furin recognition sequence, and (iii) the modified gpl20 polypeptide portion and the modified gp41 ectodomain polypeptide portion are bound to one another by a disulfide bond between the cysteine at amino acid position 511 and the cysteine at amino acid position 617.

41. The process of any of claims 1, 5, or 14, wherein the Env protein comprises (a) a first polypeptide which comprises consecutive amino acids, the sequence of which corresponds to the sequence of a modified gpl20 envelope polypeptide portion of a gpl40 envelope of an HIV-I 5768.4 isolate, or a quasi-species thereof; and (b) a second polypeptide which comprises consecutive amino acids, the sequence of which corresponds to the sequence of a modified gp41 ectodomain polypeptide portion of the gpl40 envelope of the HIV-I 5768.4 isolate or such quasi-species thereof, the sequence of said modified gpl20 envelope polypeptide portion and said modified gp41 ectodomain polypeptide portion of said HIV-I 5768.4 isolate being as set forth in SEQ
ID NO:5 and SEQ ED NO:6, respectively, said modified gp120 envelope polypeptide portion comprising a cysteine at amino acid position 519 and said modified gp41 ectodomain polypeptide portion comprising a cysteine at amino acid position 625 and a proline at amino acid position 579, wherein (i) the amino acid positions are numbered by reference to SEQ ID NO:4, (ii) the modified gp120 envelope polypeptide portion further comprises a mutated furin recognition sequence, and (iii) the modified gp120 polypeptide portion and the modified gp41 ectodomain polypeptide portion are bound to one another by a disulfide bond between the cysteine at amino acid position 519 and the cysteine at amino acid position 625.

42. The process of any of claims 1, 5, or 14, wherein the recovering further comprises purifying the trimers.

43. The process of any of claims 1, 5, or 14, wherein at least 50 µg of trimers are recovered from the preparation.

44. The process of any of claims 1, 5, or 14, further comprising formulating the recovered trimers with a pharmaceutically acceptable carrier, excipient, or diluent.
Figure 1
Figure 1 continued
Figure 2

A.

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<tr>
<th>M</th>
<th>0%</th>
<th>0.1%</th>
<th>0.05%</th>
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B.

- + Tween 20

5 min

10 min

Aggregate

Trimer
Figure 2 continued
Figure 3

Resolved in TNT-500
Figure 4 continued

- **CD4-IgG2**
- **HIVlg**

Graphs showing data with and without Tween 20.
Figure 4 continued
Figure 4 continued
Figure 5
Figure 6
Figure 7

Apparent MW = ~210k

$V = 11.30 \text{ ml}$

KNH1144

$V = 11.33 \text{ ml}$

JR-FL
Figure 8

α-2-macroglobulin

M   -   +   Tween 20
Figure 11

A

BN-PAGE

MW Standard
trimer product

669 kDa
440 kDa

B

SDS-PAGE

Reducing
Non-reducing
SeeBlue Plus 2

191 kDa
97 kDa
64 kDa
Figure 12
Figure 13
Figure 14
INTERNATIONAL SEARCH REPORT

International application No. PCT/US07/14388

A. CLASSIFICATION OF SUBJECT MATTER
IPC: A61K 39/21( 2006.01),39/00( 2006.01),39/12( 2006.01);C07K 2/00( 2006.01),1/00( 2006.01)

USPC: 424/208.1, 184.4, 186.1, 192.1;530/300,350,402,403

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S.: 424/208.1, 184.4, 186.1, 192.1;530/300, 350, 402, 403

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EAST, Dialog: Retrovirus or HIV, Env, trimer

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tr>
<td>X</td>
<td>US 6,737,067 B1 (Chevalier, M) May 18, 2004 Examples 1-3</td>
<td>1-4, 19-20, 26, 30-34, 42-44</td>
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☑ Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents:
- "X" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "Y" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "M" document published prior to the international filing date but later than the priority date claimed
- "S" document member of the same patent family

Date of the actual completion of the international search: 25 September 2007 (25.09.2007)

Date of mailing of the international search report: 03 Q4 2007

Name and mailing address of the ISA/US
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Form PCT/ISA/210 (second sheet) (April 2005)
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<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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BOX III. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-4, 26-27 (in part), and 30-44 (in part), drawn to a process for preparing retroviral trimeric Env proteins using a nonionic detergent.

Group II, claim(s) 5-13, 26-27 (in part), 28, and 30-44 (in part), drawn to a process for preparing retroviral trimeric Env proteins using column chromatography and nonionic detergent.

Group III, claim(s) 14-18, 26-27 (in part), 29, and 30-44 (in part), drawn to a process for preparing retroviral trimeric Env proteins using column chromatography and nonionic detergent.

The inventions listed as Groups I-III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The common technical feature is the presence of a nonionic detergent in a process of preparing trimeric HFV-Env proteins. Chevalier (US 6,737,067) teaches the use of Triton X-100 in the preparation of HIV-I trimeric Env proteins.
This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:
2.☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3.☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1.☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2.☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of any additional fees.
3.☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos: 
4.☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos: 1-4,26,27 and 30-44

Remark on Protest ☐ The additional search fees were accompanied by the applicant’s protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant’s protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☒ No protest accompanied the payment of additional search fees.