

TITLE OF THE INVENTION

[0001] Trimer Stabilizing HIV Envelope Protein Mutations

BACKGROUND OF THE INVENTION

[0002] Human Immunodeficiency Virus (HIV) affects millions of people worldwide, and the prevention of HIV through an efficacious vaccine remains a very high priority, even in an era of widespread antiretroviral treatment. Antigenic diversity between different strains and clades of the HIV virus renders it difficult to develop vaccines with broad efficacy. HIV-1 is the most common and pathogenic strain of the virus, with more than 90% of HIV/AIDS cases deriving from infection with HIV-1 group M. The M group is subdivided further into clades or subtypes, of which clade C is the largest. An efficacious vaccine ideally would be capable of eliciting both potent cellular responses and broadly neutralizing antibodies capable of neutralizing HIV-1 strains from different clades.

[0003] The envelope protein spike (Env) on the HIV surface is composed of a trimer of heterodimers of glycoproteins gp120 and gp41 (FIG. 1A). The precursor protein gp160 is cleaved by furin into gp120, which is the head of the spike and contains the CD4 receptor binding site as well as the large hypervariable loops (V1 to V5), and gp41, which is the membrane-anchored stem of the envelope protein spike. Like other class I fusogenic proteins, gp41 contains an N-terminal fusion peptide (FP), a C-terminal transmembrane (TM) domain, and a cytoplasmic domain. Membrane fusion between HIV and target cell membranes requires a series of conformational changes in the envelope protein. HIV vaccines can be developed based upon the envelope protein.

[0004] However, various factors make the development of an HIV vaccine based upon the envelope protein challenging, including the high genetic variability of HIV-1, the dense carbohydrate coat of the envelope protein, and the relatively dynamic and labile nature of the envelope protein spike structure. The wild-type envelope protein is unstable due to its function. Therefore, stabilizing modifications are sometimes introduced into the envelope structure for generating vaccine candidates. The envelope protein is a target for neutralizing antibodies and is highly glycosylated, which reduces the immunogenicity by shielding protein epitopes. All known broadly neutralizing antibodies (bNAbs) do accommodate these glycans.

[0005] For vaccine development, it is preferred to use envelope proteins that can induce bNAbs. However, most bNAbs only recognize the native envelope protein conformation before it undergoes any conformation changes. Therefore, developing a stable envelope protein in its native-like compact and closed conformation, while minimizing the presentation of non-native and thus non-neutralizing epitopes, could improve the efficiency of generating such bNAbs. Previous efforts to produce an HIV vaccine have focused on developing vaccines that contain the pre-fusion ectodomain of the trimeric HIV envelope protein, gp140. Gp140 does not have the transmembrane (TM) and cytoplasmic domains, but unlike gp120, it can form trimer structures. Moreover, these previous efforts have mainly focused on clade A. However, the breadth of the neutralizing antibody response that has been induced is still limited. Therefore, it would also be beneficial if stabilized native envelope trimers against multiple HIV clades were available.

[0006] For more than two decades, attempts have been made to develop a stable envelope protein in its pre-fusion trimer conformation with only limited success in producing soluble, stable trimers of the envelope protein capable of inducing a broadly neutralizing antibody response. For example, the so-called SOSIP mutations (501C, 605C and 559P) have been introduced into the envelope protein sequence to improve the formation of a soluble gp140 trimer fraction (Sanders et al., (2002), *J. Virol.* 76(17): 8875-89). The so-called SOSIP mutations include cysteine residues at positions 501 and 605, and a proline residue at position 559 according to the numbering in gp160 of HIV-1 isolate HXB2, which is the conventional numbering scheme used in the field. The introduction of the two cysteine residues at positions 501 and 605, which are close to one another in the three-dimensional protein structure results in a disulfide bridge. SOSIP mutant envelope proteins, such as BG505_SOSIP and B41_SOSIP (envelope proteins from HIV strains BG505 and B41 (i.e. 9032-08.A1.4685) strains with SOSIP mutations), have been used in vaccine studies and shown to induce tier 2 autologous neutralizing Abs (Sanders et al., *Science* (2015), 349(6224): 139-140).

[0007] However, even though the so-called SOSIP mutations are capable of stabilizing the trimer form of the envelope protein, the trimer fraction of such SOSIP mutants is usually below 10%, with large amounts of monomer and aggregates still produced. Even the SOSIP mutant BG505_SOSIP, which is one of the most promising SOSIP mutant envelope proteins known to date in terms of its ability to stabilize the trimer form typically yields up to only 25% of the trimer form (Julien et al., *Proc. Nat. Acad. Sci.* (2015), 112(38), 11947-52). Moreover, in this

trimer fraction the trimers are not completely stable as they breathe at the apex. Thus, in addition to the SOSIP mutations, several additional substitutions, such as E64K, A316W, and 201C-433C, have been designed to stabilize the apex and prevent it from breathing (de Tacye et al., *Cell* (2015), 163(7), 1702-15; Kwon et al., (2015) *Nat. Struct. Mol. Biol.* 22(7) 522-31).

[0008] Accordingly, there is a need for stabilized trimers of HIV envelope proteins that have improved percentage of trimer formation, improved trimer yield, and/or improved trimer stability. Preferably, such stabilized trimers of HIV envelope proteins would also display good binding with broadly neutralizing antibodies (bNAbs), and relatively limited binding to non-broadly neutralizing Abs (non-bNAbs). It is an object of the invention to provide HIV Env proteins that have improved trimer percentages, and preferably also improved trimer yields.

BRIEF SUMMARY OF THE INVENTION

[0009] The invention relates to recombinant HIV envelope proteins from different clades that have improved percentage of trimer formation and/or improved trimer yields as compared to previously described HIV envelope trimers. Env folding is optimized, strain-specific features are repaired, and regions of the prefusion-closed conformation important for the fusion process are stabilized by mutations described herein. This provides a universal approach to optimize the folding and stability of prefusion-closed HIV-1 envelope trimers. The resulting stable and well-folded HIV Env trimers are useful for immunization purposes, e.g. to improve chances of inducing broadly neutralizing antibodies and reducing induction of non-neutralizing and weakly neutralizing antibodies upon administration of the recombinant HIV Env trimers. The invention also relates to isolated nucleic acid molecules and vectors encoding the recombinant HIV envelope proteins, cells comprising the same, and compositions of the recombinant HIV envelope protein, nucleic acid molecule, vector, and/or cells.

[0010] In one general aspect, the invention relates to recombinant human immunodeficiency virus (HIV) envelope proteins having particular amino acid residues at identified positions in the envelope protein sequence that stabilize the formation of trimers.

[0011] In certain embodiments, a recombinant HIV envelope (Env) protein of the invention comprises the amino acid sequence of an HIV Env protein having the indicated amino acid residues at at least two of the indicated positions selected from the group consisting of:

- (i) Phe, Leu, Met, or Trp at position 651;

- (ii) Phe, Ile, Met, or Trp at position 655;
- (iii) Asn or Gln at position 535;
- (iv) Val, Ile or Ala at position 589;
- (v) Phe or Trp at position 573;
- (vi) Ile at position 204; and
- (vii) Phe, Met, or Ile at position 647,

wherein the numbering of the positions is according to the numbering in gp160 of HIV-1 isolate HXB2. In certain preferred embodiments, the indicated amino acid residue at position 651 is Phe; the indicated amino acid residue at position 655 is Ile; the indicated amino acid residue at position 535 is Asn; and/or the indicated amino acid residue at position 573 is Phe.

[0012] In certain embodiments, a recombinant HIV Env protein of the invention comprises the amino acid sequence of an HIV Env protein and an amino acid substitution by the indicated amino acid residue at at least one of the indicated positions selected from the group consisting of:

- (i) Phe, Leu, Met, or Trp at position 651;
- (ii) Phe, Ile, Met, or Trp at position 655;
- (iii) Asn or Gln at position 535;
- (iv) Val, Ile or Ala at position 589;
- (v) Phe or Trp at position 573;
- (vi) Ile at position 204; and
- (vii) Phe, Met, or Ile at position 647,

wherein the HIV Env protein is selected from the group consisting of:

- (1) an HIV Env consensus amino acid sequence, e.g. from clade C (e.g. comprising the amino acid sequence of SEQ ID NO: 2 or 3) or from clade B (e.g. comprising the amino acid sequence of SEQ ID NO: 4 or 5);
- (2) a synthetic HIV Env protein, e.g. comprising (a): the amino acid sequence of SEQ ID NO: 6, or (b): SEQ ID NO: 6 with a mutation of Glu to Arg at position 166, or (c): (a) or (b) with a mutation of the amino acids at positions 501 and 605 into Cys residues and a mutation of the amino acid at position 559 into a Pro residue, or (d): (a), (b) or (c) having a further furin cleavage site mutation, e.g. replacement of the amino acids at positions 508-511 by RRRRRR (SEQ ID NO:

- 10), or (e) SEQ ID NO: 7, or (f) a mosaic Env sequence such as Env comprising the amino acid sequence of SEQ ID NO: 8 or 9; and
- (3) a parent HIV Env protein, which preferably is a wild-type HIV Env protein, preferably of clade C, comprising at least one repair mutation at an amino acid residue that is found at the corresponding position at a frequency of less than 7.5%, preferably less than 2%, of HIV Env sequences in a collection of at least 100, preferably at least 1000, preferably at least 10000, wild-type HIV Env sequences, wherein the repair mutation is a substitution by an amino acid residue that is found at the corresponding position at a frequency of at least 10% of HIV Env sequences in said collection and preferably the repair mutation is a substitution by the amino acid residue that is found at the corresponding position most frequently in said collection;

and the numbering of the positions is according to the numbering in gp160 of HIV-1 isolate HXB2. In certain preferred embodiments, the indicated amino acid residue at position 651 is Phe; the indicated amino acid residue at position 655 is Ile; the indicated amino acid residue at position 535 is Asn; and/or the indicated amino acid residue at position 573 is Phe.

[0013] In certain embodiments, a recombinant HIV Env protein of the invention comprises the amino acid sequence of an HIV Env protein and an amino acid substitution by the indicated amino acid residue at at least one of the indicated positions selected from the group consisting of:

- (i) Phe, Leu, Met, or Trp at position 651;
- (ii) Phe, Ile, Met, or Trp at position 655;
- (iii) Asn or Gln at position 535;
- (iv) Val, Ile or Ala at position 589;
- (v) Phe or Trp at position 573;
- (vi) Ile at position 204; and
- (vii) Phe, Met, or Ile at position 647,

wherein the HIV Env protein is a SOSIP mutant HIV Env protein comprising at least one mutation resulting in the indicated amino acid residue(s) at the indicated positions selected from the group consisting of:

- (a) Cys at positions 501 and 605;
- (b) Pro at position 559; and

(c) Cys at positions 501 and 605 and Pro at position 559; and the numbering of the positions is according to the numbering in gp160 of HIV-1 isolate HXB2. In certain preferred embodiments, the indicated amino acid residue at position 651 is Phe; the indicated amino acid residue at position 655 is Ile; the indicated amino acid residue at position 535 is Asn; and the indicated amino acid residue at position 573 is Phe.

[0014] In other embodiments, a recombinant HIV Env protein of the invention further comprises an indicated amino acid residue at at least one of the indicated positions selected from the group consisting of:

- (viii) Gln, Glu, Ile, Met, Val, Trp or Phe, preferably Gln or Glu, at position 588;
- (ix) Lys at position 64, or Arg at position 66, or Lys at position 64 and Arg at position 66;
- (x) Trp at position 316;
- (xi) Cys at both positions 201 and 433;
- (xii) Pro at position 556, or Pro at position 558, or Pro at positions 556 and 558;
- (xiii) replacement of the loop at amino acid positions 548-568 (HR1-loop) by a loop having 7-10 amino acids, preferably a loop of 8 amino acids, e.g. having a sequence chosen from any one of (SEQ ID NOs: 12-17);
- (xiv) Gly at position 568, or Gly at position 569, or Gly at position 636, or Gly at both positions 568 and 636, or Gly at both positions 569 and 636; and/or
- (xv) Tyr at position 302, or Arg at position 519, or Arg at position 520, or Tyr at position 302 and Arg at position 519, or Tyr at position 302 and Arg at position 520, or Tyr at position 302 and Arg at both positions 519 and 520.

[0015] In certain embodiments, a recombinant HIV Env protein of the invention further comprises a mutation in the furin cleavage sequence of the HIV Env protein, such as a replacement at positions 508-511 by RRRRRR (SEQ ID NO: 10).

[0016] In one embodiment, the recombinant HIV Env protein is a gp140 protein.

[0017] In another embodiment, the recombinant HIV Env protein is a gp160 protein.

[0018] In certain embodiments, the recombinant HIV Env protein is truncated in the cytoplasmic region, e.g. after 7 amino acids of the cytoplasmic region.

[0019] In another general aspect, the invention relates to a trimeric complex comprising a noncovalent oligomer of three of any of the recombinant HIV Env proteins described herein.

[0020] It is another general aspect of the invention to provide a method to improve the folding and stability (measured as increased trimer percentage and/or trimer yield) of a parent HIV Env protein, the method comprising repairing the amino acid sequence of the parent HIV Env protein by introducing at least one repair mutation, preferably at least 3 repair mutations in the parent HIV Env protein, wherein a repair mutation is an amino acid substitution at an amino acid residue that is present at the corresponding position at a frequency of less than 7.5%, preferably less than 2%, of HIV Env sequences in a collection of at least 100, preferably at least 500, preferably at least 1000, preferably at least 10000, wild-type HIV Env sequences, wherein the substitution is by an amino acid residue that is present at the corresponding position at a frequency of at least 10% of HIV Env sequences in said collection and preferably the substitution is by the amino acid residue that is present at the corresponding position most frequently in said collection. The invention also provides a repaired HIV Env protein that is obtainable by said method of the invention for improving the folding and stability (measured as trimer percentage and/or trimer yield) of a HIV Env protein. The invention also provides a pharmaceutical composition comprising said repaired HIV Env protein. The invention also provides a method for producing a HIV Env protein, comprising the method for repairing the HIV Env protein described herein, and expressing nucleic acid encoding the repaired stabilized HIV Env protein in a recombinant host cell.

[0021] In another general aspect, the invention relates to a recombinant HIV Env protein comprising an amino acid sequence that is at least 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 2, wherein preferably positions 204, 535, 573, 589, 647, 651, and 655, and preferably further positions 64, 66, 201, 316, 433, 501, 508-511, 556, 558, 559, 588, 548-568 and 605 are not taken into account when determining the %identity, and wherein numbering is according to numbering in gp160 of HIV-1 isolate HXB2. In certain embodiments thereof, the recombinant HIV Env protein comprises an amino acid sequence that is at least 98%, 99% or 100% identical to SEQ ID NO: 3, wherein preferably positions 204, 535, 573, 589, 647, 651, and 655, and preferably further positions 64, 66, 201, 316, 433, 508-511, 556, 558, 588, and 548-568 are not taken into account when determining the %identity, and wherein numbering is according to numbering in gp160 of HIV-1 isolate HXB2.

In another general aspect, the invention relates to a recombinant HIV Env protein comprising an amino acid sequence that is at least 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID

NO: 4, wherein preferably positions 204, 535, 573, 589, 647, 651, and 655, and preferably further positions 64, 66, 201, 316, 433, 501, 508-511, 556, 558, 559, 588, 548-568 and 605 are not taken into account when determining the %identity, and wherein numbering is according to numbering in gp160 of HIV-1 isolate HXB2. In certain embodiments thereof, the recombinant HIV Env protein comprises an amino acid sequence that is at least 98%, 99% or 100% identical to SEQ ID NO: 5, wherein preferably positions 204, 535, 573, 589, 647, 651, and 655, and preferably further positions 64, 66, 201, 316, 433, 508-511, 556, 558, 588, and 548-568 are not taken into account when determining the %identity, and wherein numbering is according to numbering in gp160 of HIV-1 isolate HXB2.

In these aspects and embodiments, one or more of the amino acids at the indicated positions that are not taken into account for determining the %identity, are preferably chosen from the amino acids indicated as being preferred herein, e.g. Ile at position 204; Phe, Ala, Leu, or Trp at position 651; etc (see Tables 1 and 2 below).

[0022] In another general aspect, the invention relates to a recombinant HIV Env protein comprising an amino acid sequence that is at least 95%, 96%, 97%, 98%, 99% or 100% identical to any one of SEQ ID NOs: 2, 3, 4, 5, 20, 22, 24, 26, 27, 28, 29, 30, 31, or 32, wherein SEQ ID NOs: 20, 22, 24, 26, 27, 28, 29, 30, 31, or 32 are particularly preferred. In this aspect, preferably positions 204, 535, 573, 589, 647, 651, 655, and 658 and preferably further positions 64, 66, 201, 316, 433, 508-511, 556, 558, 588, and 548-568 are not taken into account when determining the %identity, and wherein numbering is according to numbering in gp160 of HIV-1 isolate HXB2. Also in this aspect, one or more of the amino acids at the indicated positions that are not taken into account for determining the %identity, are preferably chosen from the amino acids indicated as being preferred herein in (i)-(vii) of Table 1, (viii)-(xv) of Table 2, and/or (xvi) of Table 1, e.g. Ile at position 204; Phe, Leu, Met, or Trp at position 651; etc.

[0023] In another general aspect, the invention relates to a particle, preferably a liposome or nanoparticle, e.g. a self-assembling nanoparticle, displaying on its surface a recombinant HIV Env protein of the invention.

[0024] In another general aspect, the invention relates to an isolated nucleic acid molecule encoding a recombinant HIV Env protein of the invention and vectors comprising the isolated nucleic acid molecule operably linked to a promoter. In one embodiment, the vector is a viral

vector. In another embodiment, the vector is an expression vector. In one preferred embodiment, the viral vector is an adenovirus vector.

[0025] Another general aspect relates to a host cell comprising the isolated nucleic acid molecule or vector encoding the recombinant HIV Env protein of the invention. Such host cells can be used for recombinant protein production, recombinant protein expression, or the production of viral particles.

[0026] Another general aspect relates to methods of producing a recombinant HIV Env protein, comprising growing a host cell comprising an isolated nucleic acid molecule or vector encoding the recombinant HIV Env protein of the invention under conditions suitable for production of the recombinant HIV Env protein.

[0027] Yet another general aspect relates to a composition comprising a recombinant HIV Env protein, trimeric complex, isolated nucleic acid molecule, vector, or host cell as described herein, and a pharmaceutically acceptable carrier.

BRIEF DESCRIPTION OF THE FIGURES

[0028] The foregoing summary, as well as the following detailed description of the invention, will be better understood when read in conjunction with the appended drawings. It should be understood that the invention is not limited to the precise embodiments shown in the drawings.

[0029] In the figures:

[0030] FIGS. 1A and 1B show a schematic representation of the structure of HIV envelope (Env) proteins;

FIG. 1A shows a full length HIV Env protein; and

FIG. 1B shows a soluble HIV Env protein containing the so-called SOSIP mutations and a C-terminal truncation beginning at residue 664 according to the numbering in gp160 of HIV-1 isolate HXB2 (SOSIP.644 sequence);

[0031] FIGS. 2A and 2B show the percentage of trimer formation (FIG. 2A) and the trimer yield (FIG. 2B) for recombinant HIV Env proteins according to embodiments of the invention as measured by AlphaLISA assay as described in Example 3; the recombinant HIV Env proteins tested contained a single, double, or triple amino acid substitution introduced into the backbone HIV Env consensus clade C sequence ConC_SOSIP (SEQ ID NO: 3); trimer percentage and

trimer yield were determined based on the binding of the trimer specific monoclonal antibody (mAb) PGT145 to each of the recombinant HIV Env proteins; the trimer yield and percentage of trimer formation for each of the recombinant HIV Env proteins of the invention is compared to that of an envelope protein having the backbone ConC_SOSIP sequence without any additional trimer stabilizing mutations described herein;

[0032] FIG. 3 shows the chromatograms from size exclusion chromatography with multi-angle light scattering (SEC-MALS) analysis of recombinant HIV Env proteins according to embodiments of the invention; the recombinant HIV Env proteins tested contained a single amino acid substitution introduced into the backbone HIV Env consensus clade C sequence ConC_SOSIP (SEQ ID NO: 3), and were purified by lectin affinity chromatography as described in Example 2; SEC-MALS analysis was performed as described in Example 3; the peak corresponding to the trimer form is indicated in each of the chromatograms;

[0033] FIG. 4 shows the thermal stability of recombinant HIV Env proteins according to embodiments of the invention reported as percentage of trimers remaining after heat treatment; the recombinant HIV Env proteins tested contained a single, double, or triple amino acid substitution introduced into the backbone HIV Env consensus clade C sequence ConC_SOSIP (SEQ ID NO: 3); the recombinant HIV Env proteins were subjected to heat treatment, and the percentage of trimers remaining after the heat treatment was determined by AlphaLISA assay as described in Example 4; the thermal stability of the envelope protein having the backbone ConC_SOSIP sequence without any additional trimer stabilizing mutations is also shown;

[0034] FIGS. 5A-5B show the percentage of trimer formation (Fig. 5A) and the trimer yield (Fig. 5B) for recombinant HIV Env proteins having a single amino acid substitution introduced into the backbone HIV Env consensus clade B sequence ConB_SOSIP (SEQ ID NO: 5) according to embodiments of the invention compared to that of the envelope protein having the backbone ConB_SOSIP sequence without any additional trimer stabilizing mutations of the invention as described in Example 5; trimer yield and the percentage of trimer formation was measured by AlphaLISA assay;

[0035] FIGS. 6A-6B show the percentage of trimer formation and trimer yield for recombinant HIV Env proteins having amino acid substitutions introduced into the backbone synthetic HIV envelope protein sequence DS_sC4_SOSIP_E166R according to embodiments of

the invention as described in Example 6; the percentage of trimer formation and trimer yield were measured by AlphaLISA assay.

[0036] FIG. 7 shows the chromatograms from size exclusion chromatography with multi-angle light scattering (SEC-MALS) analysis of recombinant HIV Env proteins according to embodiments of the invention; the recombinant HIV Env proteins tested contained the single K655I mutation with in each next variant an additional mutation introduced into the backbone HIV Env consensus clade C sequence ConC_SOSIP (SEQ ID NO: 3), and were purified by lectin affinity chromatography as described in example 2; SEC-MALS analysis was performed as described in example 3; the peak representing the gp140 monomers in ConC_SOSIP are indicated by a shaded box, at the right side of the trimer peak. The lower panel shows a zoom in on the lower part of the graph, such that it can be seen that each additional mutation causes a further drop in the height of the gp140 monomer peak.

[0037] FIGS. 8A-8B show the percentage of trimer formation (Fig. 8A) and the trimer yield (Fig. 8B) for BG505_SOSIP (derived from a wild-type clade A strain) having single amino acid substitutions and combinations of substitutions introduced according to embodiments of the invention compared to that of the envelope protein having the backbone BG505_SOSIP sequence without any additional trimer stabilizing mutations of the invention as described in Example 9. Trimer yield and the percentage of trimer formation was measured by AlphaLISA assay.

[0038] FIG. 9 shows the chromatograms from size exclusion chromatography with multi-angle light scattering (SEC-MALS) analysis of recombinant HIV Env proteins according to embodiments of the invention; SEC-MALS analysis was performed on culture supernatant of Env transfected cells. The peak corresponding to the trimer form elutes between 7 and 7.5 minutes. The dark grey line is BG505_SOSIP (derived from a wild-type clade A strain) and the light grey line is BG505_SOSIP with L556P, K655I, M535N, N651F, D589V, K588E substitutions.

[0039] FIGS. 10A-10B show the trimer yield for C97ZA_SOSIP variants, described in Example 10. Trimer yield of C97ZA with three stabilizing substitutions (L556P, T651F and M535N) (Fig 10A and B). In Figure 10B, the Env sequence was further optimized by additional mutations (21 extra muts) that were added to repair the C97ZA Env sequence according to the conceptual framework described in FIG 12 and by introduction of additional stabilizing

substitutions (K655I, D589V, A204I and K588E). Trimer yield and the percentage of trimer formation were measured by AlphaLISA assay. The signals were normalized to ConC_SOSIP signal that was set at 1. PNGS is potential N-glycosylation site.

[0040] FIG. 11. Trimer yield of HIV-1 Env strain DU422 with four stabilizing substitutions (see Example 11 for details). All numbers were normalized to ConC_SOSIP (not shown) which was set to 1.

[0041] FIG. 12. Universal concept for repairing HIV-1 Env sequence illustrated for strain C97ZA. The residue with the highest frequency of occurrence (referred to herein as ‘consensus residue’) in the total HIV-1 database (top bars) and strain C97ZA residue (bottom bars) sorted from low to high occurrence percentage of C97ZA residue position. C97ZA sequence positions to be substituted to consensus residue were selected based on the following criteria: Positions with a C97ZA residue that occurs less than 2% in Env database sequences (black bars). Positions with a C97ZA residue that occur between 2% and 7.5% in Env database sequences and are buried or partly buried (dark grey bars). Positions that are exposed and hydrophobic in C97ZA and hydrophilic consensus residues (two lightest grey bars) and a position that is a potential N-glycosylation site (PNGS) consensus residue (S234N).

[0042] FIG. 13. Prefusion closed HIV ENV_SOSIP trimers through sequence repair and mutational stabilization. AlphaLISA signals in cell culture supernatant for all SOSIP variants normalized to the ConC_SOSIP for broadly neutralizing antibodies.

[0043] FIG. 14. Analytical SEC profile of control Env_SOSIP variants (Backbone SOSIP), repaired Env variants according to the concept described in Example 12 and FIG. 12, and Env variants with additional stabilizing substitutions according to table 3 using cell culture supernatants after transfection. Mock signal of cell culture supernatant was subtracted from all profiles. The trimer peaks are indicated with *.

[0044] FIG. 15. Trimer yield of HIV-1 Env ConC variants without the stabilizing SOSIP modifications.

[0045] FIG 16. Trimer yield (A) and trimer percentage (B) of ConC_SOSIP with mutations at positions 589, 647, 651 and 655 to methionines. All numbers were normalized to ConC_SOSIP (not shown) which was set to 1. An error bar is shown at the right end of the bars.

[0046] FIGS. 17A-17D show the percentage of trimer formation (FIG. 17A, B for different experiments) and the trimer yield (FIG. 17C, D for different experiments) for recombinant HIV

Env proteins with the indicated mutations as described in Example 15, measured by AlphaLISA assay.

[0047] FIG. 18 shows the SEC-MALS chromatograms of recombinant HIV Env proteins with the indicated mutations, as described in Example 15.

DETAILED DESCRIPTION OF THE INVENTION

[0048] Various publications, articles and patents are cited or described in the background and throughout the specification; each of these references is herein incorporated by reference in its entirety. Discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is for the purpose of providing context for the invention. Such discussion is not an admission that any or all of these matters form part of the prior art with respect to any inventions disclosed or claimed.

[0049] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention pertains. Otherwise, certain terms used herein have the meanings as set forth in the specification. All patents, published patent applications and publications cited herein are incorporated by reference as if set forth fully herein. It must be noted that as used herein and in the appended claims, the singular forms “a,” “an,” and “the” include plural reference unless the context clearly dictates otherwise.

[0050] Unless otherwise stated, any numerical values, such as a concentration or a concentration range described herein, are to be understood as being modified in all instances by the term “about.” Thus, a numerical value typically includes $\pm 10\%$ of the recited value. As used herein, the use of a numerical range expressly includes all possible subranges, all individual numerical values within that range, including integers within such ranges and fractions of the values unless the context clearly indicates otherwise.

[0051] Amino acids are referenced throughout the disclosure. There are twenty naturally occurring amino acids, as well as many non-naturally occurring amino acids. Each known amino acid, including both natural and non-natural amino acids, has a full name, an abbreviated one letter code, and an abbreviated three letter code, all of which are well known to those of ordinary skill in the art. For example, the three and one letter abbreviated codes used for the twenty

naturally occurring amino acids are as follows: alanine (Ala; A), arginine (Arg; R), aspartic acid (Asp; D), asparagine (Asn; N), cysteine (Cys; C), glycine (Gly; G), glutamic acid (Glu; E), glutamine (Gln; Q), histidine (His; H), isoleucine (Ile; I), leucine (Leu; L), lysine (Lys; K), methionine (Met; M), phenylalanine (Phe; F), proline (Pro; P), serine (Ser; S), threonine (Thr; T), tryptophan (Trp; W), tyrosine (Tyr; Y) and valine (Val; V). Amino acids can be referred to by their full name, one letter abbreviated code, or three letter abbreviated code.

[0052] Unless the context clearly dictates otherwise, the numbering of positions in the amino acid sequence of an HIV envelope protein as used herein is according to the numbering in gp160 of HIV-1 isolate HXB2 as for instance set forth in Korber et al. (Human Retroviruses and AIDS 1998: A Compilation and Analysis of Nucleic Acid and Amino Acid Sequences. Korber et al., Eds. Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, Los Alamos, N. Mex.), which is incorporated by reference herein in its entirety. Numbering according to HXB2 is conventional in the field of HIV Env proteins. The gp160 of HIV-1 isolate HXB2 has the amino acid sequence shown in SEQ ID NO: 1. Alignment of an HIV Env sequence of interest with this sequence can be used to find the corresponding amino acid numbering in the sequence of interest.

[0053] The phrases “comprises the amino acid sequence of an HIV Env protein having the indicated amino acid residue at at least one of the indicated positions selected from the group consisting of” and “comprises one or more of the following (amino acid residues)” are used interchangeably herein.

[0054] The term “percent (%) sequence identity” or “%identity” describes the number of matches (“hits”) of identical amino acids of two or more aligned amino acid sequences as compared to the number of amino acid residues making up the overall length of the amino acid sequences. In other terms, using an alignment, for two or more sequences the percentage of amino acid residues that are the same (e.g. 95%, 97% or 98% identity) may be determined, when the sequences are compared and aligned for maximum correspondence as measured using a sequence comparison algorithm as known in the art, or when manually aligned and visually inspected. The sequences which are compared to determine sequence identity may thus differ by substitution(s), addition(s) or deletion(s) of amino acids. Suitable programs for aligning protein sequences are known to the skilled person. The percentage sequence identity of protein sequences can, for example, be determined with programs such as CLUSTALW, Clustal Omega,

FASTA or BLAST, e.g. using the NCBI BLAST algorithm (Altschul SF, et al (1997), *Nucleic Acids Res.* 25:3389-3402).

[0055] A 'collection of HIV Env sequences' as used herein is a collection of a representative number (e.g. at least 100, or 500, or 1000, or more) of random sequences of wild-type HIV Env proteins, which may be from the same clade (e.g. clade C) or from different clades (e.g. clades A, B, C, etc). Suitable collections of such sequences are available in databases, or subcollections can be extracted therefrom, e.g. the HIV Sequence Database (Los Alamos National Laboratory). Such a collection comprises preferably at least 100 HIV Env protein sequences, 1000 HIV Env protein sequences, at least 10000 HIV Env protein sequences, at least 50000 HIV Env protein sequences, and may contain more than 90000 HIV Env protein sequences.

[0056] A 'corresponding position' in a HIV Env protein refers to position of the amino acid residue when at least two HIV Env sequences are aligned. Unless otherwise indicated, amino acid position numbering for these purposes is according to numbering in gp160 of HIV-1 isolate HXB2, as customary in the field.

[0057] A 'stabilizing mutation' as used herein is a mutation as described herein in any of entries (i)-(vii), or (xvi), of Table 1, or (viii)-(xv) of Table 2, which increases the percentage of trimer and/or the trimer yield (which can for instance be measured according to AlphaLISA or SEC-MALS assays described herein) of an HIV Env protein as compared to a parent molecule when the mutation is introduced by substitution of the corresponding amino acid in said parent molecule. The amino acids resulting from such stabilizing mutations typically are rarely, if at all, found in Env proteins of wild-type HIV isolates.

[0058] A 'repair mutation' as used herein is a substitution of an amino acid residue in a parent HIV Env protein, which amino acid residue is present in less than 7.5%, preferably less than 2%, at the corresponding position in a collection of HIV Env protein sequences, wherein the substitution is by an amino acid that is present at the corresponding position in said collection more frequently, e.g. in at least 10% of HIV Env proteins in said collection, and preferably is by an amino acid that is present at the corresponding position in said collection in at least 20% of HIV Env proteins or is the most frequently occurring amino acid at the corresponding position in said collection. The amino acids resulting from such repair mutations thus typically are found in a relatively high percentage of Env proteins of wild type HIV isolates, and may in several cases be the same as those at the corresponding position in consensus HIV Env sequences.

[0059] A ‘repaired and stabilized’ HIV Env sequence as used herein typically contains at least one repair mutation and at least one stabilizing mutation, preferably multiple repair mutations and multiple stabilizing mutations as compared to the parent HIV Env sequence.

[0060] The terms ‘natural’ or ‘wild-type’ are used interchangeably herein when referring to HIV strains (or Env proteins therefrom), and refer to HIV strains (or Env proteins therefrom) as occurring in nature, e.g. such as in HIV-infected patients.

[0061] The invention generally relates to recombinant HIV envelope (Env) proteins comprising certain amino acid substitutions at indicated positions in the envelope protein sequence that stabilize the trimer form of the envelope protein. Introducing one or more of the identified amino acid substitutions of the invention into the sequence of an HIV envelope protein can result in an increased percentage of trimer formation and/or an increased trimer yield. This can for instance be measured using trimer-specific antibodies, melting temperature, size exclusion chromatography, and binding to antibodies that bind to correctly folded (stable trimeric) or alternatively to incorrectly folded (non-stable or non-trimeric) Env protein, and increased trimer percentage and/or trimer yield is considered indicative of stable, native, correctly folded Env protein.

[0062] Human immunodeficiency virus (HIV) is a member of the genus *Lentivirinae*, which is part of the family of *Retroviridae*. Two species of HIV infect humans: HIV-1 and HIV-2. HIV-1 is the most common strain of HIV virus, and is known to be more pathogenic than HIV-2. As used herein, the terms “human immunodeficiency virus” and “HIV” refer to, but are not limited to, HIV-1 and HIV-2. In preferred embodiments, HIV refers to HIV-1.

[0063] HIV is categorized into multiple clades with a high degree of genetic divergence. As used herein, the term “HIV clade” or “HIV subtype” refers to related human immunodeficiency viruses classified according to their degree of genetic similarity. The largest group of HIV-1 isolates is called Group M (major strains) and consists of at least ten clades, A through J.

[0064] In one general aspect, the invention relates to a recombinant HIV envelope (Env) protein. The term “recombinant” when used with reference to a protein refers to a protein that is produced by a recombinant technique or by chemical synthesis *in vitro*. According to embodiments of the invention, a “recombinant” protein has an artificial amino acid sequence in that it contains at least one sequence element (e.g., amino acid substitution, deletion, addition, sequence replacement, etc.) that is not found in the corresponding naturally occurring sequence.

Preferably, a “recombinant” protein is a non-naturally occurring HIV envelope protein that is optimized to induce an immune response or produce an immunity against one or more naturally occurring HIV strains.

[0065] The terms “HIV envelope protein,” “HIV Env,” and “HIV Env protein” refer to a protein, or a fragment or derivative thereof, that is in nature expressed on the envelope of the HIV virion and enables an HIV to target and attach to the plasma membrane of HIV infected cells. The terms “envelope” and “Env” are used interchangeably throughout the disclosure. The HIV *env* gene encodes the precursor protein gp160, which is proteolytically cleaved into the two mature envelope glycoproteins gp120 and gp41. The cleavage reaction is mediated by a host cell protease, furin (or by furin-like proteases), at a sequence motif highly conserved in retroviral envelope glycoprotein precursors. More specifically, gp160 trimerizes to (gp160)₃ and then undergoes cleavage into the two noncovalently associated mature glycoproteins gp120 and gp41. Viral entry is subsequently mediated by a trimer of gp120/gp41 heterodimers. Gp120 is the receptor binding fragment, and binds to the CD4 receptor (and the co-receptor) on a target cell that has such a receptor, such as, e.g., a T-helper cell. Gp41, which is non-covalently bound to gp120, is the fusion fragment and provides the second step by which HIV enters the cell. Gp41 is originally buried within the viral envelope, but when gp120 binds to a CD4 receptor and co-receptor, gp120 changes its conformation causing gp41 to become exposed, where it can assist in fusion with the host cell. Gp140 is the ectodomain of gp160.

[0066] According to embodiments of the invention, an “HIV envelope (Env) protein” can be a gp160 or gp140 protein, or combinations, fusions, truncations, or derivatives thereof. For example, an “HIV envelope protein” can include a gp120 protein noncovalently associated with a gp41 protein. An “HIV envelope protein” can also be a truncated HIV envelope protein including, but not limited to, envelope proteins comprising a C-terminal truncation in the ectodomain (i.e. the domain that extends into the extracellular space), a truncation in the gp41, such as a truncation in the ectodomain of gp41, in the transmembrane domain of gp41, or a truncation in the cytoplasmic domain of gp41. An HIV envelope protein can also be a gp140, corresponding to the gp160 ectodomain, or an extended or truncated version of gp140. Expression of gp140 proteins has been described in several publications (e.g. Zhang et al., 2001; Sanders et al., 2002; Harris et al., 2011), and the protein can also be ordered from service providers, in different variants e.g. based on different HIV strains. A gp140 protein according to

the invention can have a cleavage site mutation so that the gp120 domain and gp41 ectodomain are not cleaved and covalently linked, or alternatively the gp120 domain and gp41 ectodomain can be cleaved and covalently linked, e.g. by a disulfide bridge (such as for instance in the SOSIP variants). An “HIV envelope protein” can further be a derivative of a naturally occurring HIV envelope protein having sequence mutations, e.g., in the furin cleavage sites, and/or so-called SOSIP mutations. An HIV envelope protein according to the invention can also have a cleavage site so that the gp120 and gp41 ectodomain can be non-covalently linked.

[0067] In preferred embodiments of the invention, the HIV Env protein is a gp140 protein or a gp160 protein, and more preferably a gp140 protein. In other preferred embodiments the Env protein is truncated, e.g. by deletion of the residues after the 7th residue of the cytoplasmic region as compared to a natural Env protein.

[0068] According to embodiments of the invention, an “HIV envelope protein” can be a trimer or a monomer, and is preferably a trimer. The trimer can be a homotrimer (e.g., trimers comprising three identical polypeptide units) or a heterotrimer (e.g., trimers comprising three polypeptide units that are not all identical). Preferably, the trimer is a homotrimer. In case of a cleaved gp140 or gp160, it is a trimer of polypeptide units that are gp120-gp41 dimers, and in case all three of these dimers are the same, this is considered a homotrimer.

[0069] An “HIV envelope protein” can be a soluble protein, or a membrane bound protein. Membrane bound envelope proteins typically comprise a transmembrane domain, such as in the full length HIV envelope protein comprising a transmembrane domain (TM) as shown in FIG. 1A. Membrane bound proteins can have a cytoplasmic domain, but do not require a cytoplasmic domain to be membrane bound. Soluble envelope proteins comprise at least a partial or a complete deletion of the transmembrane domain. For instance, the C-terminal end of a full length HIV envelope protein can be truncated to delete the transmembrane domain, thereby producing a soluble protein, as shown in FIG. 1B. However, the HIV envelope protein can still be soluble with shorter truncations and alternative truncation positions to those shown in FIG. 1B. Truncation can be done at various positions, and non-limiting examples are after amino acid 664, 655, 683, etc. which all result in soluble protein. A membrane-bound Env protein according to the invention may comprise a complete or a partial C-terminal domain (e.g. by partial deletion of the C-terminal cytoplasmic domain, e.g. in certain embodiments after the 7th residue of the cytoplasmic region) as compared to a native Env protein.

[0070] A signal peptide is typically present at the N-terminus of the HIV Env protein when expressed, but is cleaved off by signal peptidase and thus is not present in the mature protein. The signal peptide can be interchanged with other signal sequences, and some non-limiting examples of signal peptides are provided herein in SEQ ID NOs: 11, 18, 33, and 34.

[0071] According to embodiments of the invention, the HIV envelope protein, e.g., gp160, or gp140, can be derived from an HIV envelope protein sequence from any HIV clade (or 'subtype'), e.g., clade A, clade B, clade C, clade D, clade E, clade F, clade G, clade H, etc, or combinations thereof (such as in 'circulating recombinant forms' or CRFs derived from recombination between viruses of different subtypes, e.g BC, AE, AG, BE, BF, ADG, etc). The HIV envelope protein sequence can be a naturally occurring sequence, a mosaic sequence, a consensus sequence, a synthetic sequence, or any derivative or fragment thereof. A "mosaic sequence" contains multiple epitopes derived from at least three HIV envelope sequences of one or more HIV clades, and may be designed by algorithms that optimize the coverage of T-cell epitopes. Examples of sequences of mosaic HIV envelope proteins include those described in, e.g., Barouch et al, *Nat Med* 2010, 16: 319-323; and WO 2010/059732, such as for instance those shown in SEQ ID NOs: 8 and 9. As used herein "consensus sequence" means an artificial sequence of amino acids based on an alignment of amino acid sequences of homologous proteins, e.g. as determined by an alignment (e.g. using Clustal Omega) of amino acid sequences of homologous proteins. It is the calculated order of most frequent amino acid residues, found at each position in a sequence alignment, based upon sequences of Env from at least 1000 natural HIV isolates. A "synthetic sequence" is a non-naturally occurring HIV envelope protein that is optimized to induce an immune response or produce immunity against more than one naturally occurring HIV strains. Mosaic HIV envelope proteins are non-limiting examples of synthetic HIV envelope proteins. In preferred embodiments of the invention, the HIV Env protein is a consensus Env protein, or a synthetic Env protein, having at least one of the indicated amino acids at the indicated positions (i)-(vii) according to the invention. Particularly preferred are consensus Env proteins having at least one, preferably at least two of the indicated amino acid residues at the indicated positions (i)-(vii) according to the invention, preferably having further SOSIP and/or furin cleavage site mutations as described below.

[0072] In certain embodiments of the invention, an HIV envelope protein, whether a naturally occurring sequence, mosaic sequence, consensus sequence, synthetic sequence etc.,

comprises additional sequence mutations e.g., in the furin cleavage sites, and/or so-called SOSIP mutations.

[0073] In some embodiments of the invention, an HIV envelope protein is a “SOSIP mutant HIV Env protein.” The so-called SOSIP mutations are trimer stabilizing mutations that include the ‘SOS mutations’ (Cys residues at positions 501 and 605, which results in the introduction of a possible disulfide bridge between the newly created cysteine residues) and the ‘IP mutation’ (Pro residue at position 559). According to embodiments of the invention, a SOSIP mutant Env protein comprises at least one mutation selected from the group consisting of Cys at positions 501 and 605; Pro at position 559; and preferably Cys at positions 501 and 605 and Pro at position 559. A SOSIP mutant HIV Env protein can further comprise other sequence mutations, e.g., in the furin cleavage site. In addition, in certain embodiments it is possible to further add mutations such that the Env protein comprises Pro at position 556 or position 558 or at positions 556 and 558, which were found herein to be capable of acting not only as alternatives to Pro at position 559 in a SOSIP variant, but also as additional mutations that could further improve trimer formation of a SOSIP variant that already has Pro at position 559.

[0074] In certain preferred embodiments of the invention, a SOSIP mutant HIV Env protein comprises Cys at positions 501 and 605, and Pro at position 559.

[0075] In certain embodiments, an HIV envelope protein of the invention further comprises a mutation in the furin cleavage site. The mutation in the furin cleavage sequence can be an amino acid substitution, deletion, insertion, or replacement of one sequence with another, or replacement with a linker amino acid sequence. Preferably in the present invention, mutating the furin cleavage site can be used to optimize the cleavage site, so that furin cleavage is improved over wild-type, for instance by a replacement of the sequence at residues 508-511 with RRRRRR (SEQ ID NO: 10) [i.e. replacement of a typical amino acid sequence (e.g. EK) at positions 509-510 with four arginine residues (i.e. two replacements and two additions), while at positions 508 and 511, there are already arginine residues present in most HIV Env proteins, so these typically do not need to be replaced, but since the end result in literature is often referred to as amino acid sequence RRRRRR, we kept this nomenclature herein]. Other mutations that improve furin-cleavage are known and can also be used. Alternatively, it is possible to replace the furin cleavage site with a linker, so that furin cleavage is no longer necessary but the protein will adopt a native-like conformation (e.g. described in (Sharma et al, 2015) and (Georgiev et al, 2015)).

[0076] In particular embodiments of the invention, an HIV envelope protein of the invention further comprises both the so-called SOSIP mutations (preferably Cys at positions 501 and 605, and Pro at position 559) and a sequence mutation in the furin cleavage site, preferably a replacement of the sequence at residues 508-511 with RRRRRR (SEQ ID NO: 10). In certain preferred embodiments, the HIV Env comprises both the indicated SOSIP and furin cleavage site mutations, and in addition further comprises a Pro residue at position 556 or 558, most preferably at both positions 556 and 558.

[0077] In preferred embodiments of the invention, the amino acid sequence of the HIV envelope protein is a consensus sequence, such as an HIV envelope clade C consensus or an HIV envelope clade B consensus. In a particularly preferred embodiment, the amino acid sequence of the HIV envelope protein is an HIV envelope clade C consensus.

[0078] Exemplary HIV envelope proteins that can be used in the invention include HIV envelope clade C consensus (SEQ ID NO: 2) and HIV envelope clade B consensus (SEQ ID NO: 4). These HIV envelope clade C and clade B consensus sequences can comprise additional mutations that, e.g., enhance stability and/or trimer formation, such as for instance the so-called SOSIP mutations and/or a sequence mutation in the furin cleavage site as described above, such as for instance in the ConC_SOSIP sequence shown in SEQ ID NO: 3 and the ConB_SOSIP sequence shown in SEQ ID NO: 5.

[0079] Other non-limiting examples of preferred HIV envelope protein sequences that can be used in the invention (as 'background' or 'parent' molecule, wherein then one or more of the mutations of the invention are introduced) include synthetic HIV Env proteins, for instance comprising the amino acid sequence of SEQ ID NO: 6, or SEQ ID NO: 6 with a mutation of Glu to Arg at position 166, either of those optionally having further SOSIP and/or furin cleavage site mutations as described above. Another non-limiting example is SEQ ID NO: 7. Further non-limiting examples are mosaic HIV envelope proteins, such as those having the amino acid sequence of SEQ ID NO: 8 or 9.

[0080] In certain embodiments, the parent molecule is a wild-type HIV Env protein, wherein one or preferably more amino acids have been repaired according to methods described herein. Such parent molecules comprise at least one repair mutation at an amino acid residue that is present at the corresponding position at a frequency of less than 7.5%, preferably less than 2%, of HIV Env sequences in a collection of at least 100, preferably at least 500, preferably at least

1000, preferably at least 10000, preferably at least 20000, wild-type HIV Env sequences, wherein the repair mutation is a substitution by an amino acid residue that is present at the corresponding position at a frequency of at least 10% of HIV Env sequences in said collection. Preferably said substitution is by an amino acid residue that is present at the corresponding position at a frequency of at least 15%, at least 20%, at least 25%, of HIV Env sequences in said collection. Preferably, said substitution is by the amino acid residue that is present at the corresponding position most frequently in said collection. In certain preferred embodiments, said parent molecules comprise at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or at least 20 of such repair mutations. Preferably at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or all of the amino acid residues that are present at the corresponding positions at a frequency of less than 2% of HIV Env sequences in said collection are repaired in the parent molecule as compared to the wild-type Env protein. In certain embodiments, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or all of the amino acid residues that are present at the corresponding positions at a frequency of less than 7.5% of HIV Env sequences in said collection are repaired in the parent molecule as compared to the wild-type Env protein. In certain embodiments, the wild-type HIV Env protein is from a clade A, B, or C strain, preferably from a clade C strain. As a result of this repairing mutations, the parent molecule will show more resemblance to a HIV Env consensus sequence than the original wild-type strain, hence the repaired amino acid residue is sometimes referred to herein as 'consensus amino acid' or 'consensus residue'. The result of this repair activity is greatly enhanced properties of the resulting parent molecule with respect to folding, trimerization, expression, and/or stability, and the resulting molecule is referred to herein as a 'repaired Env protein'. The addition of the stabilizing mutations of the invention (e.g. one or more of (i)-(vii) (Table 1), and/or (xvi) (Table 1), and/or optionally (viii)-(xv) (Table 2), into such parent molecules leads to an even further improvement in one or more of trimer percentage, trimer yield, stability, broadly neutralizing antibody binding, folding, and the resulting molecules that are derived from wild-type HIV Env proteins are referred to herein as 'repaired and stabilized Env protein'. It will be clear to the skilled person that introduction of the stabilizing mutations actually diverts the resulting sequence a bit from a consensus sequence, so the net result of greatly enhanced properties of repaired and stabilized HIV Env molecules is based on two entirely different concepts.

[0081] Mutations resulting in the indicated amino acids at positions (i)-(vii) according to the present invention can also be used in HIV Env proteins wherein no SOSIP mutations are present (e.g. in Env consensus sequences or in Env proteins from wild-type HIV isolates) and are likely to also improve the trimerization thereof, as the mutations of the invention are independent from the SOSIP mutations, and in addition were shown to work in several different HIV Env protein backbones. Indeed, it is shown herein that mutations according to the invention can work in the absence of the SOS-mutations as well as in the absence of the IP-mutation to improve HIV Env trimerization properties.

[0082] A recombinant HIV envelope protein according to embodiments of the invention comprises an HIV envelope protein having certain amino acid residue(s) at specified positions in the amino acid sequence of an HIV envelope protein. In particular, seven positions in the envelope protein were identified, as well as the particular amino acid residues to be desirable at each of the identified positions. The identified positions in the envelope protein sequence include (i) position 651, (ii) position 655, (iii) position 535, (iv) position 589, (v) position 573, (vi) position 204, and (vii) position 647, wherein the numbering of the positions is according to the numbering in gp160 of HIV-1 isolate HXB2. An HIV Env protein according to the invention has the specified amino acid residue(s) in at least one of the indicated positions (i)-(vii), preferably at at least two of the indicated positions (i)-(vii), more preferably at at least three of the indicated positions (i)-(vii). The particular amino acid residues that are desirable to be at each of the identified positions according to embodiments of the invention are shown in Table 1. The preferred positions of these options are (i), (ii), (iii), (iv), (vi), and/or (vii). Particularly preferred positions of these options are (i), (ii), (iii), (iv), and/or (vii). An additional preferred option is (xvi), mentioned in some more detail later herein.

[0083] Table 1: Desirable Amino Acids at Indicated Positions in the Recombinant HIV Env Proteins According to Embodiments of the Invention

<u>No.</u>	<u>Position¹</u>	<u>Desirable Amino Acid Residue</u>
(i)	651	Phe, Leu, Met, or Trp (preferably Phe)
(ii)	655	Phe, Ile, Met, or Trp (preferably Ile)
(iii)	535	Asn or Gln (preferably Asn)

(iv)	589	Val, Ile, or Ala (preferably Val or Ile, most preferably Val)
(v)	573	Phe or Trp (preferably Phe)
(vi)	204	Ile
(vii)	647	Phe, Met, or Ile (preferably Phe)
(xvi)	658	Val, Ile, Phe, Met, Ala, or Leu (preferably Val or Ile, most preferably Val)

¹ According to the numbering in gp160 of HIV-1 isolate HXB2

[0084] The amino acid sequence of the HIV envelope protein into which one or more desirable amino acid (or indicated amino acid) substitutions at the one or more indicated positions are introduced is referred to as the “backbone HIV envelope sequence” or “parent HIV envelope sequence.” For example, if position 651 in the ConC_SOSIP sequence of SEQ ID NO: 3 is mutated to Phe, then the ConC_SOSIP sequence is considered to be the “backbone” or “parent” sequence. Any HIV envelope protein can be used as the “backbone” or “parent” sequence into which a novel stabilizing mutation according to an embodiment of the invention can be introduced, either alone or in combination with other mutations, such as the so-called SOSIP mutations and/or mutations in the furin cleavage site. Non-limiting examples of HIV Env protein that could be used as backbone include HIV Env protein from a natural HIV isolate, a synthetic HIV Env protein, or a consensus HIV Env protein, and in certain non-limiting examples include those comprising SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, or SEQ ID NO: 9.

[0085] According to embodiments of the invention, the HIV envelope protein can have the indicated amino acid residue at at least one of the indicated positions selected from the group consisting of positions 651, 655, 535, 589, 573, 204, and 647, such as the indicated amino acid residue at one, two, three, four, five, six, or seven positions. Preferably, the HIV envelope protein is substituted at one, two or three of the indicated positions, and more preferably the HIV envelope protein is substituted at at least two of the indicated positions. Even more preferably, the HIV Env protein is substituted at three of the indicated positions, four of the indicated positions, five of the indicated positions, six of the indicated positions, or all seven of the indicated positions. Preferably, the HIV envelope protein contains the indicated amino acid residues at at least two of the indicated positions. More preferably, the HIV envelope protein

contains the indicated amino acid residues at three of the indicated positions. In other preferred embodiments, the HIV envelope protein contains the indicated amino acid residues at four, five, six, or all seven of the indicated positions.

[0086] Embodiments of HIV Env proteins having the indicated amino acids at multiple positions are (positions numbered according to numbering in gp160 of HIV-1 isolate HXB2 followed by one letter amino acid code for the residue present on that position, positions within one HIV Env protein embodiment divided by commas [e.g. an embodiment of an Env protein having Phe at position 651 and Ile at position 655 is described as 651F, 655I], while different embodiments (i.e. different HIV Env proteins) are divided by semicolons) include the following. For Env proteins with the indicated amino acids at two positions: 651F, 655I; 651F, 655F; 651F, 655M; 651F, 655W; 651F, 535N; 651F, 535Q; 651F, 589V; 651F, 589I; 651F, 589A; 651F, 573F; 651F, 573W; 651F, 204I; 651F, 647F; 651F, 647I; 651F, 647M; 651L, 655I; 651L, 655F; 651L, 655M; 651L, 655W; 651L, 535N; 651L, 535Q; 651L, 589V; 651L, 589I; 651L, 589A; 651L, 573F; 651L, 573W; 651L, 204I; 651L, 647F; 651L, 647I; 651L, 647M; 651M, 655I; 651M, 655F; 651M, 655A; 651M, 655L; 651M, 655W; 651M, 535N; 651M, 535Q; 651M, 589V; 651M, 589I; 651M, 589A; 651M, 573F; 651M, 573W; 651M, 204I; 651M, 647F; 651M, 647I; 651M, 647M; 651W, 655I; 651W, 655F; 651W, 655M; 651W, 655W; 651W, 535N; 651W, 535Q; 651W, 589V; 651W, 589I; 651W, 589A; 651W, 573F; 651W, 573W; 651W, 204I; 651W, 647F; 651W, 647I; 651W, 647M; 655I, 535N; 655I, 535Q; 655I, 589V; 655I, 589I; 655I, 589A; 655I, 573F; 655I, 573W; 655I, 204I; 655I, 647F; 655I, 647I; 655I, 647M; 655F, 535N; 655F, 535Q; 655F, 589V; 655F, 589I; 655F, 589A; 655F, 573F; 655F, 573W; 655F, 204I; 655F, 647F; 655F, 647I; 655F, 647M; 655M, 535N; 655M, 535Q; 655M, 589V; 655M, 589I; 655M, 589A; 655M, 573F; 655M, 573W; 655M, 204I; 655M, 647F; 655M, 647I; 655M, 647M; 655W, 535N; 655W, 535Q; 655W, 589V; 655W, 589I; 655W, 589A; 655W, 573F; 655W, 573W; 655W, 204I; 655W, 647F; 655W, 647I; 655W, 647M; 535N, 589V; 535N, 589I; 535N, 589A; 535N, 573F; 535N, 573W; 535N, 204I; 535N, 647F; 535N, 647I; 535N, 647M; 535Q, 589V; 535Q, 589I; 535Q, 589A; 535Q, 573F; 535Q, 573W; 535Q, 204I; 535Q, 647F; 535Q, 647I; 535Q, 647M; 589V, 573F; 589V, 573W; 589V, 204I; 589V, 647F; 589V, 647I; 589V, 647M; 589I, 573F; 589I, 573W; 589I, 204I; 589I, 647F; 589I, 647I; 589I, 647M; 589A, 573F; 589A, 573W; 589A, 204I; 589A, 647F; 589A, 647I; 589A, 647M; 573F, 204I; 573F, 647F; 573F, 647I; 573F, 647M; 573W, 204I; 573W, 647F; 573W, 647I; 573W, 647M; 204I, 647F;

204I, 647I; 201I, 647M. Each of those embodiments can be present according to the invention in any HIV Env sequence, such as a wild-type isolate, or a SOSIP mutant HIV Env protein, or a consensus HIV Env protein, or a synthetic HIV Env protein. Each of those embodiments can be combined with one of the preferred amino acids according to the invention at a third position of one of the other indicated positions from (i)-(vii) according to the invention. Such embodiments, having preferred amino acid residues at three positions of the indicated positions (i)-(vii) can be combined with one of the preferred amino acids at a fourth position of one of the other indicated positions from (i)-(vii) according to the invention. Such embodiments, having preferred amino acid residues at four positions of the indicated positions (i)-(vii) can be combined with one of the preferred amino acids at a fifth position of one of the other indicated positions from (i)-(vii) according to the invention. Such embodiments, having preferred amino acid residues at five positions of the indicated positions (i)-(vii) can be combined with one of the preferred amino acids at a sixth position of one of the other indicated positions from (i)-(vii) according to the invention. Such embodiments, having preferred amino acid residues at six positions of the indicated positions (i)-(vii) can be combined with one of the preferred amino acids at a seventh position of one of the other indicated positions from (i)-(vii) according to the invention, such that the Env protein has a preferred amino acid according to the invention at all seven positions (i)-(vii) according to the invention. Any of these further embodiments having preferred amino acids according to the invention at three, four, five, six or seven of the positions (v)-(vii) of the invention, can be present in any HIV Env protein, such as from a wild-type isolate, a SOSIP variant, a consensus HIV Env protein, a synthetic HIV Env protein, and the like.

[0087] Preferred Env proteins according to the invention with the indicated amino acids at two positions are: 651F, 655I; 651F, 535N; 651F, 589V; 651F, 589I; 651F, 573F; 651F, 204I; 651F, 647F; 655I, 535N; 655I, 589V; 655I, 589I; 655I, 573F; 655I, 204I; 655I, 647F; 535N, 589V; 535N, 589I; 535N, 573F; 535N, 204I; 535N, 647F ; 589V, 573F; 589V, 204I; 589V, 647F; 589I, 573F; 589I, 204I; 589I, 647F; 573F, 204I; 573F, 647F; 204I, 647F. Particularly preferred Env proteins having preferred amino acids at at least two positions according to the invention include: 651F, 655I; 655I, 535N; 655I, 589V; 535N, 589V; 535N, 647F.

[0088] Some preferred HIV Env proteins having preferred amino acid residues at three positions are: 651F, 655I, 535N; 651F, 589V, 535N; 651F, 589I, 535N; 651F, 573F, 535N;

651F, 204I, 535N; 651F, 647F, 535N; 655I, 589V, 535N; 655I, 589I, 535N; 655I, 573F, 535N; 655I, 204I, 535N; 655I, 647F, 535N; 589V, 573F, 535N; 589V, 204I, 535N; 589V, 647F, 535N; 589I, 573F, 535N; 589I, 204I, 535N; 589I, 647F, 535N; 573F, 204I, 535N; 573F, 647F, 535N; 204I, 647F, 535N; 651F, 655I, 589V; 651F, 573F, 589V; 651F, 204I, 589V; 651F, 647F, 589V; 655I, 573F, 589V; 655I, 204I, 589V; 655I, 647F, 589V; 573F, 204I, 589V; 573F, 647F, 589V; 204I, 647F, 589V; 651F, 655I, 589I; 651F, 573F, 589I; 651F, 204I, 589I; 651F, 647F, 589I; 655I, 573F, 589I; 655I, 204I, 589I; 655I, 647F, 589I; 573F, 204I, 589I; 573F, 647F, 589I; 204I, 647F, 589I; 651F, 655I, 573F; 651F, 204I, 573F; 651F, 647F, 573F; 655I, 204I, 573F; 655I, 647F, 573F; 204I, 647F, 573F; 651F, 655I, 204I; 651F, 647F, 204I; 655I, 647F, 204I; 651F, 655I, 647F; 655I, 651F, 647F; 655I, 651F, 535N; 655I, 589V, 573F; 655I, 589V, 204I.

Particularly preferred Env proteins having preferred amino acids at at least three positions according to the invention include: 651F, 655I, 535N; 655I, 589V, 535N; 655I, 573F, 589V; 655I, 204I, 589V; 651F, 655I, 647F.

[0062] Some preferred HIV Env proteins having preferred amino acid residues at four positions are: 651F, 655I, 535N, 589V; 651F, 655I, 535N, 573F; 651F, 655I, 589V, 573F; 651F, 535N, 589V, 573F; 655I, 535N, 589V, 573F; 651F, 655I, 535N, 204I; 651F, 655I, 589V, 204I; 651F, 535N, 589V, 204I; 655I, 535N, 589V, 204I; 651F, 655I, 573F, 204I; 651F, 535N, 573F, 204I; 655I, 535N, 573F, 204I; 651F, 589V, 573F, 204I; 655I, 589V, 573F, 204I; 535N, 589V, 573F, 204I; 651F, 655I, 535N, 647F; 651F, 655I, 589V, 647F; 651F, 535N, 589V, 647F; 655I, 535N, 589V, 647F; 651F, 655I, 573F, 647F; 651F, 535N, 573F, 647F; 655I, 535N, 573F, 647F; 651F, 589V, 573F, 647F; 655I, 589V, 573F, 647F; 535N, 589V, 573F, 647F; 651F, 655I, 204I, 647F; 651F, 535N, 204I, 647F; 655I, 535N, 204I, 647F; 651F, 589V, 204I, 647F; 655I, 589V, 204I, 647F; 535N, 589V, 204I, 647F; 651F, 573F, 204I, 647F; 655I, 573F, 204I, 647F; 535N, 573F, 204I, 647F; and 589V, 573F, 204I, 647F.

Some examples of preferred HIV Env proteins having preferred amino acid residues at at least four positions include: 651F, 655I, 647F, 535N; 651F, 655I, 573F, 589V. A preferred example of an HIV Env protein comprising the indicated amino acid residues at at least four positions comprises 535N, 589V, 651F, 655I. Non-limiting examples of such HIV Env proteins are provided in SEQ ID NOs: 20, 22, 24, 26, 27, 28, 29, 30, 31, and 32. Preferably such HIV Env protein is a clade C HIV Env protein or a clade A HIV Env protein, most preferably a clade C HIV Env protein. In certain embodiments, said HIV Env protein further comprises 588E, i.e. it

comprises at least 535N, 588E, 589V, 651F, 655I. Non-limiting examples of such HIV Env protein are provided in SEQ ID NOs: 20, 24, 26, 27, 28, 29, 30, 31, and 32. In certain embodiments, said HIV Env further comprises 556P, i.e. it comprises at least 535N, 556P, 589V, 651F, 655I or at least 535N, 556P, 588E, 589V, 651F, 655I. Non-limiting examples of such HIV Env protein are provided in SEQ ID NOs: 22, 24, 26, 27, 29, 30, 31 and 32.

[0089] In one embodiment, a recombinant HIV Env protein according to the invention comprises the amino acid sequence of an HIV Env protein having the indicated amino acid residues at at least two of the indicated positions selected from the group consisting of:

- (i) Phe, Leu, Met, or Trp at position 651;
- (ii) Phe, Ile, Met, or Trp at position 655;
- (iii) Asn or Gln at position 535;
- (iv) Val, Ile, or Ala at position 589;
- (v) Phe or Trp at position 573;
- (vi) Ile at position 204; and
- (vii) Phe, Met, or Ile at position 647.

For example, the recombinant HIV Env protein can have one of Phe, Leu, Met, or Trp at position 651, and Asn or Gln at position 535, optionally, additional indicated amino acid residues at the additional indicated positions. Preferably, at least one of the amino acids in (i)-(vii) is introduced into the recombinant HIV Env protein by amino acid substitution. For example, the recombinant HIV Env protein can be produced from an HIV Env protein that contains none or only one of the amino acid residues in (i)-(vii) above such that all or one or more of the at least two indicated amino acid residues are introduced into the recombinant HIV Env protein by amino acid substitution.

[0090] In certain embodiments, the recombinant HIV Env protein of the invention further comprises (viii) Gln, Glu, Ile, Met, Val, Trp, or Phe at position 588, wherein Gln or Glu are preferred.

[0091] The amino acid sequence of the HIV Env protein into which the above described substitutions are introduced can be any HIV Env protein known in the art in view of the present disclosure, such as, for instance a naturally occurring sequence from HIV clade A, clade B, clade C, etc.; a mosaic sequence; a consensus sequence, e.g., clade B or clade C consensus sequence; a synthetic sequence; or any derivative or fragment thereof. In certain embodiments of the

invention, the amino acid sequence of the HIV Env protein comprises additional mutations, such as, for instance, the so-called SOSIP mutations, and/or a mutation in the furin cleavage site.

[0092] In one particular embodiment, the HIV Env backbone protein is a SOSIP mutant HIV Env protein comprising at least one mutation selected from the group consisting of Cys at positions 501 and 605; Pro at position 559. In a preferred embodiment, the SOSIP mutant HIV Env protein comprises Cys at positions 501 and 605, and Pro at position 559. According to this embodiment, a recombinant HIV Env protein comprises the amino acid sequence of the SOSIP mutant HIV Env protein and an amino acid substitution by the indicated amino acid residue at at least one of the indicated positions selected from the group consisting of:

- (i) Phe, Leu, Met, or Trp at position 651;
- (ii) Phe, Ile, Met, or Trp at position 655;
- (iii) Asn or Gln at position 535;
- (iv) Val, Ile, or Ala at position 589;
- (v) Phe or Trp at position 573;
- (vi) Ile at position 204; and
- (vii) Phe, Met, or Ile at position 647.

The SOSIP mutant HIV Env protein can further comprise a mutation in the furin cleavage site, such as a replacement at positions 608-511 by SEQ ID NO: 10.

[0093] In another embodiment, a recombinant HIV Env protein according to the invention comprises the amino acid sequence of an HIV Env protein and an amino acid substitution by the indicated amino acid residue at at least one of the indicated positions selected from the group consisting of:

- (i) Phe, Leu, Met, or Trp at position 651;
- (ii) Phe, Ile, Met, or Trp at position 655;
- (iii) Asn or Gln at position 535;
- (iv) Val, Ile, or Ala at position 589;
- (v) Phe or Trp at position 573;
- (vi) Ile at position 204; and
- (vii) Phe, Met, or Ile at position 647,

wherein the HIV Env protein is selected from the group consisting of:

- (1) an HIV Env consensus sequence, such as a clade C or clade B consensus sequence, e.g. comprising the amino acid sequence of SEQ ID NO: 2, 3, 4 or 5;
- (2) a synthetic HIV Env protein, e.g. comprising the amino acid sequence of (a) SEQ ID NO: 6; (b) SEQ ID NO: 6 with a mutation of Glu to Arg at position 166; (c) SEQ ID NO: 7; (d) SEQ ID NO: 8 or 9, (a), (b) or (d) optionally having further SOSIP and/or furin cleavage site mutations as described above.

Preferably, the recombinant HIV Env protein comprises the amino acid sequence of an HIV Env protein and an amino acid substitution by the indicated amino acid residue at at least two of the indicated positions selected from the group consisting of (i)-(vii) above, such as two positions or three positions. However, the recombinant HIV Env protein can comprise an amino acid substitution by the indicated amino acid residue at one or more of the indicated positions, such as one, two, three, four, five, six, or seven of the indicated positions.

[0094] In one particular embodiment, the HIV Env backbone protein is an HIV Env consensus clade C comprising an amino acid sequence that is at least 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 2. Preferably, the HIV consensus clade C sequence of SEQ ID NO: 2 further comprises the so-called SOSIP mutations, i.e., Cys at positions 501 and 605, and Pro at position 559, and more preferably further comprises the so-called SOSIP mutations and a mutation in the furin cleavage site, such as for instance a replacement at positions 508-511 by SEQ ID NO: 10. In a particularly preferred embodiment, the HIV Env backbone protein comprises the sequence shown in SEQ ID NO: 3, or a sequence at least 95% identical thereto, wherein preferably amino acids at positions 501, 559, 605, and 508-511 as replaced by SEQ ID NO: 10, are not mutated as compared to SEQ ID NO: 3.

[0095] In another particular embodiment, the HIV Env backbone protein is an HIV Env consensus clade B comprising an amino acid sequence that is at least 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 4. Preferably, the HIV consensus clade B sequence of SEQ ID NO: 4 further comprises the so-called SOSIP mutations, i.e., Cys at positions 501 and 605, and Pro at position 559, and more preferably further comprises the so-called SOSIP mutations and a mutation in the furin cleavage site, such as for instance a replacement at positions 508-511 by SEQ ID NO: 10. In a particularly preferred embodiment, the HIV Env backbone protein comprises the sequence shown in SEQ ID NO: 5, or a sequence at

least 95% identical thereto, wherein preferably amino acids at positions 501, 559, 605, and 508-511 as replaced by SEQ ID NO: 10, are not mutated as compared to SEQ ID NO: 5.

[0096] In yet another particular embodiment, the HIV Env backbone protein is a synthetic HIV Env protein, e.g. comprising the amino acid sequence of (a) SEQ ID NO: 6; (b) SEQ ID NO: 6 with a mutation of Glu to Arg at position 166; (c) SEQ ID NO: 7; or (d) SEQ ID NO: 8 or 9, (a) (b) or (d) optionally having further SOSIP (501C, 605C, 559P) and/or furin cleavage site mutations (508-511RRRRRR) as described above.

[0097] In yet other particular embodiments, the HIV Env backbone protein is a HIV Env protein from a wild-type clade A or clade C HIV virus, optionally comprising mutations to repair the sequence according to methods described herein.

[0098] Exemplary combinations of two positions in the HIV Env protein that can be simultaneously substituted include residues 535,589; 535,647; and 589,655; such as for instance in double mutants I535N, D589V; I535N, E647F; and D589V, K655I. Other double mutants include K655I, I535N; N651F, K655I; and K655I, I573F. An exemplary combination of three positions in the HIV Env protein that can be simultaneously substituted includes 535,589,655, such as for instance in triple mutant I535N, D589V, K655I. Other triple mutants include K655I, D589V, I573F; and K655I, N651F, I535N.

[0099] In certain embodiments of the invention, a recombinant HIV Env protein according to the invention can further comprise an indicated amino acid residue (e.g. via substitution) at one or more additional indicated positions selected from the group consisting of positions (viii) 588, (ix) 64 or 66, (x) 316, (xi) 201/433, (xii) 556 or 558 or 556 and 558, (xiii) 548-568, (xiv) 568, 569 and 636, or (xv) 302, 519 or 520, as shown in Table 2 below. Certain of these amino acid substitutions (e.g. (viii)) were found by the present inventors to combine very well with (combinations of) mutations (i)-(vii) according to the invention as described above. Other of these amino acid substitutions have been previously reported in the literature. For example, De Taeye et al. (*Cell* (2015) 163(7), 1702-15) reported an HIV envelope protein having an E64K and T316W double mutation, and an HIV Env protein having a 66R mutation; and Kwon et al. (*Nat. Struct. Mol. Biol.* (2015) 22(7) 522-31) reported an HIV envelope protein having an I204C, A433C disulfide substitution; and Guenaga et al. (*Immunity* (2017) 46, 792-803) reported an HIV envelope protein having L568G, T569G or N636G, and N302Y, F519R, L520R triple substitution. However, to the best of the knowledge of the inventors, these previously described

mutations were not described in combination with any of the novel substitutions described herein, e.g., the substitutions listed in Table 1. These amino acid mutations in combination with the amino acid substitutions of the invention can further increase trimer yield and/or the percentage of trimer formation. These amino acid substitutions can be introduced into any of the recombinant HIV Env proteins described herein in addition to substitution by the indicated amino acid residue at one or more of the indicated positions as described in Table 1.

Table 2: Additional Positions of Amino Acid Substitution and Residue of Substitution

No.	Position ¹	Indicated Amino Acid Residue
(viii)	588	Gln, Glu, Ile, Met, Val, Trp, or Phe (preferably Gln or Glu)
(ix)	64 or 66	Lys at position 64; or Arg at position 66
(x)	316	Trp
(xi)	201 and 433	Cys at both positions
(xii)	556 or 558 or 556 and 558	Pro at either or both positions
(xiii)	548-568 (HR1 loop)	Replacement by shorter and less flexible loop having 7-10 amino acids, preferably a loop of 8 amino acids, e.g. having a sequence chosen from any one of (SEQ ID NOs: 12-17)
(xiv)	568, 569, 636	Gly at any one of these positions, or Gly at both positions 568 and 636, or Gly at both positions 569 and 636
(xv)	302, 519, 520	Tyr at position 302, or Arg at position 519, or Arg at position 520; or Tyr at position 302 and Arg at position 519; or Tyr at position 302 and Arg at position 520; or Tyr at position 302 and Arg at both positions 519 and 520

¹ According to the numbering in gp160 of HIV-1 isolate HXB2

The substitutions identified at the indicated positions of the present invention [(i)-(vii), see e.g. Table 1] are not or rarely present in natural sequences, are not found in combination in previously reported HIV Env protein sequences, and were not previously suggested to result in improved trimerization of the HIV Env protein, improved trimer yield and/or increased trimer stability. The mutations (ix)-(xi) in Table 2 (that were previously reported by others) are all in the gp120 region, to which the trimer specific antibody PGT145 binds. These mutations keep the trimer closed at the apex (which is at the top of the molecule). The substitutions (xii) and (xiii) are all in the HR1 of gp41. Except for position 204, the mutations of the present invention in Table 1 are all in the gp41 region (at the bottom part of the molecule), but outside the HR1

region. Clearly, the previously described mutations did not provide any suggestion for introduction of the mutations of the present invention, let alone the surprising effects thereof on trimer formation with a closed apex as measured by PGT145 binding. Apart from the point mutations (viii)-(xii) in Table 2, it is also possible to replace the HR1 loop of the Env protein (amino acid residues 548-568 in a wild-type sequence, with numbering according to gp160 of the HXB2 isolate) by a shorter and less flexible loop having 7-10 amino acids, preferably a loop of 8 amino acids, e.g. having a sequence chosen from any one of (SEQ ID NOs: 12-17), see e.g. Kong et al (Nat Commun. 2016 Jun 28;7:12040. doi: 10.1038/ncomms12040) that describes such shorter loops replacing the HR1 loop. Such an Env variant, further having the indicated amino acid residues at at least one and preferably at at least two of the indicated positions (i)-(vii) according to the invention, is also an embodiment of the invention. Mutations listed in (viii)-(xiii) can in certain embodiments of the invention be added to HIV Env proteins of the invention, i.e. having one or more of the indicated amino acids at positions (i)-(vii). Also, combinations within the groups (viii)-(xiii) can be made, a non-limiting example being a combination of mutations (in addition to at least one mutation of (i)-(vii)) at (viii) and (xii) (e.g. I535N, A558P, K588E). Some non-limiting examples of double mutants that were made in an HIV Env background with SOSIP mutations and combining mutations at at least one of positions (i)-(vii) and at at least one of positions (viii)-(xiii) include: 535,588; 588,589; 655,588; 558,535; and 655,556; such as for instance I535N, K588E; 588Q, D589V; K655I, K588E; A558P, I535N; and K655I, L556P. Some non-limiting examples of such triple mutants include 558,535,588; 558,535,589; 558,535,655; and 558,535,651, such as for instance A558P, I535N, K588E; A558P, I535, D589V; A558P, I535N, K655I; and A558P, I535N, N651F.

Further non-limiting examples of combinations according to the invention include 655I, 573F, 589V, 588E; 651F, 655I, 573F, 589V, 588E; 651F, 655I, 573F, 589V, 588E, 535N; 651F, 655I, 573F, 589V, 588E, 535N, 204I; 651F, 655I, 556P; 651F, 535N, 556P; 651F, 589V, 556P; 651F, 589I, 556P; 651F, 573F, 556P; 651F, 204I, 556P; 651F, 647F, 556P; 655I, 535N, 556P; 655I, 589V, 556P; 655I, 589I, 556P; 655I, 573F, 556P; 655I, 204I, 556P; 655I, 647F, 556P; 535N, 589V, 556P; 535N, 589I, 556P; 535N, 573F, 556P; 535N, 204I, 556P; 535N, 647F, 556P; 589V, 573F, 556P; 589V, 204I, 556P; 589V, 647F, 556P; 589I, 573F, 556P; 589I, 204I, 556P; 589I, 647F, 556P; 573F, 204I, 556P; 573F, 647F, 556P; 651F, 655I, 558P; 651F, 535N, 558P; 651F, 589V, 558P; 651F, 589I, 558P; 651F, 573F, 558P; 651F, 204I, 558P; 651F, 647F, 558P;

655I, 535N, 558P; 655I, 589V, 558P; 655I, 589I, 558P; 655I, 573F, 558P; 655I, 204I, 558P; 655I, 647F, 558P; 535N, 589V, 558P; 535N, 589I, 558P; 535N, 573F, 558P; 535N, 204I, 558P; 535N, 647F, 558P; 589V, 573F, 558P; 589V, 204I, 558P; 589V, 647F, 558P; 589I, 573F, 558P; 589I, 204I, 558P; 589I, 647F, 558P; 573F, 204I, 558P; 573F, 647F, 558P; 655I, 589V, 535N, 556P; 651F, 655I, 535N, 556P; 556P, 651F; 556P, 651F, 655I, 535N; 655I, 589V, 573F, 651F, 588E, 556P; 556P, 651F, 655I, 535N, 573F; 556P, 651F, 655I, 535N, 573F, 589V; 556P, 651F, 655I, 535N, 573F, 589V, 204I; 556P, 651F, 655I, 535N, 573F, 589V, 204I, 588Q; 556P, 651F, 655I, 535N, 573F, 589V, 204I, 588Q, 647F; 556P, 651F, 535N, 573F; 556P, 651F, 535N, 573F, 589V; 556P, 651F, 535N, 573F, 589V, 204I; 556P, 651F, 535N, 573F, 589V, 204I, 588Q; 556P, 651F, 535N, 573F, 589V, 204I, 588Q, 647F; 556P, 655I, 535N, 573F; 556P, 655I, 535N, 573F, 589V; 556P, 655I, 535N, 573F, 589V, 204I; 556P, 655I, 535N, 573F, 589V, 204I, 588Q; 556P, 655I, 535N, 573F, 589V, 204I, 588Q, 647F. Again, any of those embodiments can be in any HIV Env protein, e.g. a wild-type isolate, a consensus Env, a synthetic Env protein, a SOSIP mutant Env protein, a wild-type isolate containing repair mutations according to the concept described herein, etc. Some preferred combinations according to the invention include 655I, 589V, 573F, 651F, 588E, 535N, 204I; 556P, 655I, 535N, 573F, 589V, 204I, 588Q; 204I, 535N, 556P, 588E, 589V, 651F, 655I; 535N, 556P, 589V, 651F, 655I; and 535N, 556P, 588E, 589V, 651F, 655I.

In certain preferred embodiments, the HIV Env protein comprises a sequence that is at least 95% identical to, preferably at least 96%, 97%, 98%, 99% identical to, preferably 100% identical to, any one of SEQ ID NOs: 20, 22, 24, 26, 27, 28, 29, 30, 31 and 32. For determination of the %identity, preferably the positions (i)-(xv) of Tables 1 and 2, and preferably also positions 501, 559 and 605 are not taken into account. Preferably the amino acid residues at those positions are the ones in the sequences of SEQ ID NO: 20, 22, 24, 26, 27, 28, 29, 30, 31 or 32, respectively.

[00100] In certain embodiments, the HIV Env protein of the invention further comprises: (xvi) an amino acid residue chosen from Val, Ile, Phe, Met, Ala, or Leu at position 658. Preferably the amino acid at position 658 is Val or Ile, most preferably Val. It was found that this strongly increased trimer percentage and trimer yield of the Env protein, either alone or in combination with mutations chosen from (i)-(vii) of Table 1 and/or (viii)-(xv) of Table 2 described herein.

[00101] According to embodiments of the invention, a recombinant HIV Env protein has at least one of (a) an improved percentage of trimer formation and (b) an improved trimer yield

compared to an HIV Env protein not having the indicated amino acid residues at one or more of positions 651, 655, 535, 589, 573, 204, and 647 as shown in Table 1.

[00102] As used herein “improved percentage of trimer formation” means that a greater percentage of trimer is formed when the backbone sequence of the HIV envelope protein contains one or more of the amino acid substitutions of the invention as compared to the percentage of trimer that is formed when the backbone sequence of the HIV envelope sequence does not contain such amino acid substitutions. As used herein “improved trimer yield” means that a greater total amount of the trimer form of the envelope protein is obtained when the backbone sequence of the HIV envelope protein contains one or more of the amino acid substitutions of the invention as compared to the total amount of trimer form of the envelope protein that is obtained when the backbone sequence of the HIV envelope sequence does not contain such amino acid substitutions.

[00103] Trimer formation can be measured by an antibody binding assay using antibodies that bind specifically to the trimer form of the HIV Env protein. Examples of trimer specific antibodies that can be used to detect the trimer form include, but are not limited to, the monoclonal antibodies (mAbs) PGT145, PGDM1400, PG16, and PGT151. Preferably, the trimer specific antibody is mAb PGT145. Any antibody binding assay known in the art in view of the present disclosure can be used to measure the percentage of trimer formation of a recombinant HIV Env protein of the invention, such as ELISA, AlphaLISA, etc.

[00104] In a particular embodiment, trimer formation is measured by AlphaLISA. AlphaLISA is a bead-based proximity assay in which singlet oxygen molecules, generated by high energy irradiation of donor beads, are transferred to acceptor beads that are within a distance of approximately 200 nm with respect to the donor beads. The transfer of singlet oxygen molecules to the acceptor beads initiates a cascading series of chemical reactions resulting in a chemiluminescent signal that can then be detected (Eglen et al. *Curr. Chem. Genomics*, 2008, 25(1): 2-10). For example, recombinant HIV envelope proteins labeled with a Flag-His tag can be incubated with a trimer specific mAb, donor beads conjugated to the antibody that binds to the trimer specific mAb, nickel-conjugated donor beads, acceptor beads conjugated to an anti-His antibody, and acceptor beads conjugated to an anti-Flag antibody. The amount of trimer formed can be determined by measuring the chemiluminescent signal generated from the pair of donor beads conjugated to the antibody that binds to the trimer specific mAb and

the acceptor beads conjugated to the anti-His antibody. The total amount of HIV envelope protein expressed can be determined by measuring the chemiluminescent signal generated from the pair of nickel-conjugated donor beads and anti-Flag-conjugated acceptor beads. For example, the amount of trimer and the total envelope protein expressed can be measured by an AlphaLISA assay as described in detail in Example 3. The percentage of trimer formation can be calculated by dividing the amount of trimer formed by the total amount of expressed envelope protein.

[00105] The amount of trimer formed and the total amount of envelope protein expressed can also be determined using chromatographic techniques that are capable of separating the trimer form from other forms of the HIV envelope protein, e.g., the monomer form. Examples of such techniques that can be used include, but are not limited to size exclusion chromatography multi-angle light scattering (SEC-MALS). According to certain embodiments, the percentage of trimer formation is determined using SEC-MALS. According to certain embodiments, the trimer yield is determined using SEC-MALS.

[00106] Nucleic Acid, Vectors, and Cells

[00107] In another general aspect, the invention provides a nucleic acid molecule encoding a recombinant HIV Env protein according to the invention, and a vector comprising the nucleic acid molecule. The nucleic acid molecules of the invention can be in the form of RNA or in the form of DNA obtained by cloning or produced synthetically. The DNA can be double-stranded or single-stranded. The DNA can for example comprise cDNA, genomic DNA, or combinations thereof. The nucleic acid molecules and vectors can be used for recombinant protein production, expression of the protein in a host cell, or the production of viral particles.

[00108] According to embodiments of the invention, the nucleic acid encoding the recombinant HIV envelope protein is operably linked to a promoter, meaning that the nucleic acid is under the control of a promoter. The promoter can be a homologous promoter (i.e., derived from the same genetic source as the vector) or a heterologous promoter (i.e., derived from a different vector or genetic source). Examples of suitable promoters include the human cytomegalovirus immediate early (hCMV IE, or shortly “CMV”) promoter and the Rous Sarcoma virus (RSV) promoter. Preferably, the promoter is located upstream of the nucleic acid within an expression cassette.

[00109] According to embodiments of the invention, a vector can be an expression vector. Expression vectors include, but are not limited to, vectors for recombinant protein expression and vectors for delivery of nucleic acid into a subject for expression in a tissue of the subject, such as a viral vector. Examples of viral vectors suitable for use with the invention include, but are not limited to adenoviral vectors, adeno-associated virus vectors, pox virus vectors, Modified Vaccinia Ankara (MVA) vectors, enteric virus vectors, Venezuelan Equine Encephalitis virus vectors, Semliki Forest Virus vectors, Tobacco Mosaic Virus vectors, lentiviral vectors, etc. The vector can also be a non-viral vector. Examples of non-viral vectors include, but are not limited to plasmids, bacterial artificial chromosomes, yeast artificial chromosomes, bacteriophages, etc.

[00110] In certain embodiments of the invention, the vector is an adenovirus vector, e.g., a recombinant adenovirus vector. A recombinant adenovirus vector may for instance be derived from a human adenovirus (HAdV, or AdHu), or a simian adenovirus such as chimpanzee or gorilla adenovirus (ChAd, AdCh, or SAdV) or rhesus adenovirus (rhAd). Preferably, an adenovirus vector is a recombinant human adenovirus vector, for instance a recombinant human adenovirus serotype 26, or any one of recombinant human adenovirus serotype 5, 4, 35, 7, 48, etc. In other embodiments, an adenovirus vector is a rhAd vector, e.g. rhAd51, rhAd52 or rhAd53.

[00111] The preparation of recombinant adenoviral vectors is well known in the art. For example, preparation of recombinant adenovirus 26 vectors is described, in, e.g., WO 2007/104792 and in Abbink *et al.*, (2007) *Virol.* 81(9): 4654-63. Exemplary genome sequences of adenovirus 26 are found in GenBank Accession EF 153474 and in SEQ ID NO: 1 of WO 2007/104792. Exemplary genome sequences for rhAd51, rhAd52 and rhAd53 are provided in US 2015/0291935.

[00112] According to embodiments of the invention, any of the recombinant HIV Env proteins described herein can be expressed and/or encoded by any of the vectors described herein. In view of the degeneracy of the genetic code, the skilled person is well aware that several nucleic acid sequences can be designed that encode the same protein, according to methods entirely routine in the art. The nucleic acid encoding the recombinant HIV Env protein of the invention can optionally be codon-optimized to ensure proper expression in the host cell (e.g., bacterial or mammalian cells). Codon-optimization is a technology widely applied in the art.

[00113] The invention also provides cells, preferably isolated cells, comprising any of the nucleic acid molecules and vectors described herein. The cells can for instance be used for recombinant protein production, or for the production of viral particles.

[00114] Embodiments of the invention thus also relate to a method of making a recombinant HIV Env protein. The method comprises transfecting a host cell with an expression vector comprising nucleic acid encoding a recombinant HIV Env protein according to an embodiment of the invention operably linked to a promoter, growing the transfected cell under conditions suitable for expression of the recombinant HIV Env protein, and optionally purifying or isolating the recombinant HIV Env protein expressed in the cell. The recombinant HIV Env protein can be isolated or collected from the cell by any method known in the art including affinity chromatography, size exclusion chromatography, etc. Techniques used for recombinant protein expression will be well known to one of ordinary skill in the art in view of the present disclosure. The expressed recombinant HIV Env protein can also be studied without purifying or isolating the expressed protein, e.g., by analyzing the supernatant of cells transfected with an expression vector encoding the recombinant HIV Env protein and grown under conditions suitable for expression of the HIV Env protein.

[00115] In a preferred embodiment, the expressed recombinant HIV Env protein is purified under conditions that permit association of the protein so as to form the stabilized trimeric complex. For example, mammalian cells transfected with an expression vector encoding the recombinant HIV Env protein operably linked to a promoter (e.g. CMV promoter) can be cultured at 33-39°C, e.g. 37°C, and 2-12% CO₂, e.g. 8% CO₂. Expression can also be performed in alternative expression systems such as insect cells or yeast cells, all conventional in the art. The expressed HIV Env protein can then be isolated from the cell culture for instance by lectin affinity chromatography, which binds glycoproteins. The HIV Env protein bound to the column can be eluted with mannopyranoside. The HIV Env protein eluted from the column can be subjected to further purification steps, such as size exclusion chromatography, as needed, to remove any residual contaminants, e.g., cellular contaminants, but also Env aggregates, gp140 monomers and gp120 monomers. Alternative purification methods, non-limiting examples including antibody affinity chromatography, negative selection with non-bNAbs, anti-tag purification, or other chromatography methods such as ion exchange chromatography etc, as well as other methods known in the art, could also be used to isolate the expressed HIV Env protein.

[00116] The nucleic acid molecules and expression vectors encoding the recombinant HIV Env proteins of the invention can be made by any method known in the art in view of the present disclosure. For example, nucleic acid encoding the recombinant HIV Env protein can be prepared by introducing at least one of the amino acid substitutions at the indicated positions into the backbone HIV envelope sequence using genetic engineering technology and molecular biology techniques, e.g., site directed mutagenesis, polymerase chain reaction (PCR), etc., which are well known to those skilled in the art. The nucleic acid molecule can then be introduced or “cloned” into an expression vector also using standard molecular biology techniques. The recombinant HIV envelope protein can then be expressed from the expression vector in a host cell, and the expressed protein purified from the cell culture by any method known in the art in view of the present disclosure.

[00117] Trimeric Complex

[00118] In another general aspect, the invention relates to a trimeric complex comprising a noncovalent oligomer of three of the recombinant HIV Env proteins according to the invention. The trimeric complex can comprise any of the recombinant HIV Env proteins described herein. Preferably the trimeric complex comprises three identical monomers (or identical heterodimers if gp140 is cleaved) of the recombinant HIV Env proteins according to the invention. The trimeric complex can be separated from other forms of the HIV envelope protein, such as the monomer form, or the trimeric complex can be present together with other forms of the HIV envelope protein, such as the monomer form.

[00119] Compositions and Methods

[00120] In another general aspect, the invention relates to a composition comprising a recombinant HIV Env protein, trimeric complex, isolated nucleic acid, vector, or host cell, and a pharmaceutically acceptable carrier. The composition can comprise any of the recombinant HIV Env proteins, trimeric complexes, isolated nucleic acid molecules, vectors, or host cells described herein.

[00121] A carrier can include one or more pharmaceutically acceptable excipients such as binders, disintegrants, swelling agents, suspending agents, emulsifying agents, wetting agents, lubricants, flavorants, sweeteners, preservatives, dyes, solubilizers and coatings. The precise nature of the carrier or other material can depend on the route of administration, e.g., intramuscular, intradermal, subcutaneous, oral, intravenous, cutaneous, intramucosal (e.g., gut),

intranasal or intraperitoneal routes. For liquid injectable preparations, for example, suspensions and solutions, suitable carriers and additives include water, glycols, oils, alcohols, preservatives, coloring agents and the like. For solid oral preparations, for example, powders, capsules, caplets, gelcaps and tablets, suitable carriers and additives include starches, sugars, diluents, granulating agents, lubricants, binders, disintegrating agents and the like. For nasal sprays/inhalant mixtures, the aqueous solution/suspension can comprise water, glycols, oils, emollients, stabilizers, wetting agents, preservatives, aromatics, flavors, and the like as suitable carriers and additives.

[00122] Compositions of the invention can be formulated in any matter suitable for administration to a subject to facilitate administration and improve efficacy, including, but not limited to, oral (enteral) administration and parenteral injections. The parenteral injections include intravenous injection or infusion, subcutaneous injection, intradermal injection, and intramuscular injection. Compositions of the invention can also be formulated for other routes of administration including transmucosal, ocular, rectal, long acting implantation, sublingual administration, under the tongue, from oral mucosa bypassing the portal circulation, inhalation, or intranasal.

[00123] Embodiments of the invention also relate to methods of making the composition. According to embodiments of the invention, a method of producing a composition comprises mixing a recombinant HIV Env protein, trimeric complex, isolated nucleic acid, vector, or host cell of the invention with one or more pharmaceutically acceptable carriers. One of ordinary skill in the art will be familiar with conventional techniques used to prepare such compositions.

[00124] HIV antigens (e.g., proteins or fragments thereof derived from HIV *gag*, *pol*, and/or *env* gene products) and vectors, such as viral vectors, expressing the HIV antigens have previously been used in immunogenic compositions and vaccines for vaccinating a subject against an HIV infection, or for generating an immune response against an HIV infection in a subject. As used herein, “subject” means any animal, preferably a mammal, most preferably a human, to who will be or has been administered an immunogenic composition according to embodiments of the invention. The term “mammal” as used herein, encompasses any mammal. Examples of mammals include, but are not limited to, mice, rats, rabbits, guinea pigs, monkeys, humans, etc., preferably a human. The recombinant HIV Env proteins of the invention can also be used as antigens to induce an immune response against human immunodeficiency virus (HIV) in a subject in need thereof. The immune response can be against one or more HIV clades, such

as clade A, clade B, clade C, etc. The compositions can comprise a vector from which the recombinant HIV Env protein is expressed, or the composition can comprise an isolated recombinant HIV Env protein according to an embodiment of the invention.

[00125] For example, compositions comprising a recombinant HIV protein or a trimeric complex thereof can be administered to a subject in need thereof to induce an immune response against an HIV infection in the subject. A composition comprising a vector, such as an adenovirus vector, encoding a recombinant HIV Env protein of the invention, wherein the recombinant HIV Env protein is expressed by the vector, can also be administered to a subject in need thereof to induce an immune response against an HIV infection in the subject. The methods described herein also include administering a composition of the invention in combination with one or more additional HIV antigens (e.g., proteins or fragments thereof derived from HIV *gag*, *pol*, and/or *env* gene products) that are preferably expressed from one or more vectors, such as adenovirus vectors or MVA vectors, including methods of priming and boosting an immune response.

[0126] In certain embodiments, the HIV Env protein can be displayed on a particle, such as a liposome, virus-like particle (VLP), nanoparticle, virosome, or exosome, optionally in combination with endogenous and/or exogenous adjuvants. When compared to soluble or monomeric Env protein on its own, such particles typically display enhanced efficacy of antigen presentation *in vivo*.

Examples of VLPs that display HIV Env protein can be prepared e.g. by co-expressing the HIV Env protein with self-assembling viral proteins such as HIV Gag core or other retroviral Gag proteins. VLPs resemble viruses, but are non-infectious because they contain no viral genetic material. The expression of viral structural proteins, such as envelope or capsid, can result in self-assembly of VLPs. VLPs are well known to the skilled person, and their use in vaccines is for instance described in (Kushnir et al, 2012).

In certain preferred embodiments, the particle is a liposome. A liposome is a spherical vesicle having at least one lipid bilayer. The HIV Env trimer proteins can for instance be non-covalently coupled to such liposomes by electrostatic interactions, e.g. by adding a His-tag to the C-terminus of the HIV Env trimer and a bivalent chelating atom such as Ni^{2+} or Co^{2+} incorporated into the head group of derivatized lipids in the liposome. In certain non-limiting and exemplary embodiments, the liposome comprises 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC),

cholesterol, and the Nickel or Cobalt salt of 1,2-dioleoyl-*sn*-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (DGS-NTA(Ni²⁺) or DGS-NTA(Co²⁺)) at 60:36:4 molar ratio. In preferred embodiments, the HIV Env trimer proteins are covalently coupled to the liposomal surface, e.g. via a maleimide functional group integrated in the liposome surface. In certain non-limiting exemplary embodiments thereof, the liposome comprises DSPC, cholesterol, and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-[4-(p-maleimidomethyl)cyclohexanecarboxamide] lipid in a molar ratio of 54:30:16. The HIV Env protein can be coupled thereto e.g. via an added C-terminal cysteine in the HIV Env protein. The covalently coupled variants are more stable, elicit high antigen specific IgG titers and epitopes at the antigenically less relevant 'bottom' of the Env trimer are masked. Methods for preparing HIV Env trimers coupled to liposomes, as well as their characterization, are known and have for instance been described in (Bale et al, 2017), incorporated by reference herein. The invention also provides an HIV Env protein of the invention fused to and/or displayed on a liposome.

In certain embodiments, a HIV Env protein of the invention is fused to self-assembling particles, or displayed on nanoparticles. Antigen nanoparticles are assemblies of polypeptides that present multiple copies of antigens, e.g. the HIV Env protein of the instant invention, which result in multiple binding sites (avidity) and can provide improved antigen stability and immunogenicity. Preparation and use of self-assembling protein nanoparticles for use in vaccines is well-known to the skilled person, see e.g. (Zhao et al, 2014), (López-Sagaseta et al, 2016). As non-limiting examples, self-assembling nanoparticles can be based on ferritin, bacterioferritin, or DPS. DPS nanoparticles displaying proteins on their surface are for instance described in WO2011/082087. Description of trimeric HIV-1 antigens on such particles has for instance been described in (He et al, 2016). Other self-assembling protein nanoparticles as well as preparation thereof, are for instance disclosed in WO 2014/124301, and US 2016/0122392, incorporated by reference herein. The invention also provides an HIV Env protein of the invention fused to and/or displayed on a self-assembling nanoparticle. The invention also provides compositions comprising VLPs, liposomes, or self-assembling nanoparticles according to the invention.

[0127] In certain embodiments, an adjuvant is included in a composition of the invention or co-administered with a composition of the invention. Use of adjuvant is optional, and may further enhance immune responses when the composition is used for vaccination purposes. Adjuvants suitable for co-administration or inclusion in compositions in accordance with the

invention should preferably be ones that are potentially safe, well tolerated and effective in people. Such adjuvants are well known to the skilled person, and non-limiting examples include QS-21, Detox-PC, MPL-SE, MoGM-CSF, TiterMax-G, CRL- 1005, GERBU, TERamide, PSC97B, Adjuvax, PG-026, GSK-I, GcMAF, B-aethine, MPC-026, Adjuvax, CpG ODN, Betafectin, Aluminium salts such as Aluminium Phosphate (e.g. AdjuPhos) or Aluminium Hydroxide, and MF59.

[0128] Other aspects of the invention relate to recombinant HIV envelope proteins comprising an amino acid sequence that is at least 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 4, which represent the HIV envelope consensus clade C and consensus clade B sequences, respectively. These consensus sequences have not been found in any naturally occurring sequences, and are thus believed to be novel HIV envelope proteins. A recombinant HIV envelope protein comprising an amino acid sequence that is at least 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 4 can optionally further comprise the so-called SOSIP mutations and/or a mutation in the furin cleavage site, such as, for instance in those sequences shown in SEQ ID NO: 3, or SEQ ID NO: 3 further comprising Pro at position 558 and/or position 556; and SEQ ID NO: 5, or SEQ ID NO: 5 further comprising Pro at position 558 and/or position 556. When determining the %identity for these sequences, the amino acids at the mutated furin cleavage site and at positions 501, 605, 559, 556 and 558 are preferably not taken into account. It was surprisingly found that such proteins are expressed at high levels and have a high level of stability and trimer formation. Such HIV Env proteins can in certain embodiments be used as backbone proteins, wherein the mutations described above can be made to obtain a molecule of the invention. Isolated nucleic acid molecules encoding these sequences, vectors comprising these sequences operably linked to a promoter, and compositions comprising the protein, isolated nucleic acid molecule, or vector are also contemplated by the invention.

EMBODIMENTS

[0129] Embodiment 1 is a recombinant HIV Env protein, comprising the amino acid sequence of an HIV Env protein having the indicated amino acid residues at at least two of the indicated positions selected from the group consisting of:

- (i) Phe, Leu, Met, or Trp, preferably Phe, at position 651;

- (ii) Phe, Ile, Met, or Trp, preferably Ile, at position 655;
- (iii) Asn or Gln, preferably Asn, at position 535;
- (iv) Val, Ile or Ala, preferably Val or Ile, at position 589;
- (v) Phe or Trp, preferably Phe, at position 573;
- (vi) Ile at position 204; and
- (vii) Phe, Met, or Ile, preferably Phe, at position 647,

wherein the numbering of the positions is according to the numbering in gp160 of HIV-1 isolate HXB2.

[0130] Embodiment 2 is a recombinant HIV Env protein, comprising the amino acid sequence of an HIV Env protein and an amino acid substitution by the indicated amino acid residue at at least one of the indicated position selected from the group consisting of:

- (i) Phe, Leu, Met, or Trp, preferably Phe, at position 651;
- (ii) Phe, Ile, Met, or Trp, preferably Ile, at position 655;
- (iii) Asn or Gln, preferably Asn, at position 535;
- (iv) Val, Ile or Ala, preferably Val or Ile, at position 589;
- (v) Phe or Trp, preferably Phe at position 573;
- (vi) Ile at position 204; and
- (vii) Phe, Met, or Ile, preferably Phe, at position 647,

wherein the HIV Env protein is selected from the group consisting of:

- (1) an HIV Env protein having a consensus sequence, for instance from clade C or from clade B, e.g. comprising the amino acid sequence of (SEQ ID NO: 2, 3, 4 or 5); or
- (2) a synthetic HIV Env protein, for instance comprising the amino acid sequence of (a) SEQ ID NO: 6; (b) SEQ ID NO: 6 with a mutation of Glu to Arg at position 166; (c) SEQ ID NO: 7; or (d) SEQ ID NO: 8 or SEQ ID NO: 9), wherein (a), (b) or (d) optionally may have further SOSIP (Cys at positions 501 and 605 and Pro at position 559) and/or furin cleavage site mutations (e.g. SEQ ID NO: 10 replacing amino acids 508-511); or
- (3) a wild-type HIV Env protein, preferably of clade C, comprising at least one repair mutation at an amino acid residue that is present at the corresponding position at a frequency of less than 7.5%, preferably less than 2%, of HIV Env sequences in a

collection of at least 100, preferably at least 1000, preferably at least 10000, wild-type HIV Env sequences, wherein the repair mutation is a substitution by an amino acid residue that is present at the corresponding position at a frequency of at least 10% of HIV Env sequences in said collection and preferably the repair mutation is a substitution by the amino acid residue that is present at the corresponding position most frequently in said collection; and

wherein the numbering of the positions is according to the numbering in gp160 of HIV-1 isolate HXB2.

[0131] Embodiment 3 is a recombinant HIV Env protein, comprising the amino acid sequence of an HIV Env protein and an amino acid substitution by the indicated amino acid residue at at least one of the indicated position selected from the group consisting of:

- (i) Phe, Leu, Met, or Trp, preferably Phe, at position 651;
- (ii) Phe, Ile, Met, or Trp, preferably Ile, at position 655;
- (iii) Asn or Gln, preferably Asn, at position 535;
- (iv) Val, Ile or Ala, preferably Val or Ile, at position 589;
- (v) Phe or Trp, preferably Phe, at position 573;
- (vi) Ile at position 204; and
- (vii) Phe, Met, or Ile, preferably Phe, at position 647,

wherein the HIV Env protein is a SOSIP mutant HIV Env protein comprising at least one mutation selected from the group consisting of:

- (a) Cys at positions 501 and 605;
- (b) Pro at position 559;
- (c) Cys at positions 501 and 605 and Pro at position 559; and

the numbering of the positions is according to the numbering in gp160 of HIV-1 isolate HXB2.

[0132] Embodiment 4 is the recombinant HIV Env protein of embodiment 2, comprising the indicated amino acid residues at at least two of the indicated positions selected from the group consisting of (i) to (vii).

[0133] Embodiment 5 is the recombinant HIV Env protein of embodiment 3, comprising the indicated amino acid residues at at least two of the indicated positions selected from the group consisting of (i) to (vii).

[0134] Embodiment 6 is the recombinant HIV Env protein of any of embodiments 1, 2 and 4, further comprising Cys at positions 501 and 605 or Pro at position 559, preferably Cys at positions 501 and 605 and Pro at position 559.

[0135] Embodiment 7 is the recombinant HIV Env protein of any of embodiments 1 to 6, comprising the indicated amino acid residues at at least three of the indicated positions selected from the group consisting of (i) to (vii).

[0136] Embodiment 8 is the recombinant HIV Env protein of any of embodiments 1 to 6, comprising the indicated amino acid residues at at least four of the indicated positions selected from the group consisting of (i) to (vii).

[0137] Embodiment 9 is the recombinant HIV Env protein of any of embodiments 1 to 6, comprising the indicated amino acid residues at at least five of the indicated positions selected from the group consisting of (i) to (vii).

[0138] Embodiment 10 is the recombinant HIV Env protein of any of embodiments 1 to 6, comprising the indicated amino acid residues at at least six of the indicated positions selected from the group consisting of (i) to (vii).

[0139] Embodiment 11 is the recombinant HIV Env protein of any of embodiments 1 to 6, comprising the indicated amino acid residues at seven of the indicated positions selected from the group consisting of (i) to (vii).

[0140] Embodiment 12 is the recombinant HIV Env protein of any of embodiments 1, 4, and 5, wherein the at least two indicated positions and residues are a combination selected from the group consisting of: 651F, 655I; 651F, 535N; 651F, 589V; 651F, 589I; 651F, 573F; 651F, 204I; 651F, 647F; 655I, 535N; 655I, 589V; 655I, 589I; 655I, 573F; 655I, 204I; 655I, 647F; 535N, 589V; 535N, 589I; 535N, 573F; 535N, 204I; 535N, 647F ; 589V, 573F; 589V, 204I; 589V, 647F; 589I, 573F; 589I, 204I; 589I, 647F; 573F, 204I; 573F, 647F; and 204I, 647F.

[0141] Embodiment 13 is the recombinant HIV Env protein of embodiment 7, wherein the at least three indicated positions and residues are a combination selected from the group consisting of: 651F, 655I, 535N; 651F, 589V, 535N; 651F, 589I, 535N; 651F, 573F, 535N; 651F, 204I, 535N; 651F, 647F, 535N; 655I, 589V, 535N; 655I, 589I, 535N; 655I, 573F, 535N; 655I, 204I, 535N; 655I, 647F, 535N; 589V, 573F, 535N; 589V, 204I, 535N; 589V, 647F, 535N; 589I, 573F, 535N; 589I, 204I, 535N; 589I, 647F, 535N; 573F, 204I, 535N; 573F, 647F, 535N; 204I, 647F, 535N; 651F, 655I, 589V; 651F, 573F, 589V; 651F, 204I, 589V; 651F, 647F, 589V; 655I, 573F,

589V; 655I, 204I, 589V; 655I, 647F, 589V; 573F, 204I, 589V; 573F, 647F, 589V; 204I, 647F, 589V; 651F, 655I, 589I; 651F, 573F, 589I; 651F, 204I, 589I; 651F, 647F, 589I; 655I, 573F, 589I; 655I, 204I, 589I; 655I, 647F, 589I; 573F, 204I, 589I; 573F, 647F, 589I; 204I, 647F, 589I; 651F, 655I, 573F; 651F, 204I, 573F; 651F, 647F, 573F; 655I, 204I, 573F; 655I, 647F, 573F; 204I, 647F, 573F; 651F, 655I, 204I; 651F, 647F, 204I; 655I, 647F, 204I; 651F, 655I, 647F; 655I, 651F, 647F; 655I, 651F, 535N; 655I, 589V, 573F; and 655I, 589V, 204I.

[0142] Embodiment 14 is the recombinant HIV Env protein of embodiment 8, wherein the at least four indicated positions and residues are a combination selected from the group consisting of: 651F, 655I, 535N, 589V; 651F, 655I, 535N, 573F; 651F, 655I, 589V, 573F; 651F, 535N, 589V, 573F; 655I, 535N, 589V, 573F; 651F, 655I, 535N, 204I; 651F, 655I, 589V, 204I; 651F, 535N, 589V, 204I; 655I, 535N, 589V, 204I; 651F, 655I, 573F, 204I; 651F, 535N, 573F, 204I; 655I, 535N, 573F, 204I; 651F, 589V, 573F, 204I; 655I, 589V, 573F, 204I; 535N, 589V, 573F, 204I; 651F, 655I, 535N, 647F; 651F, 655I, 589V, 647F; 651F, 535N, 589V, 647F; 655I, 535N, 589V, 647F; 651F, 655I, 573F, 647F; 651F, 535N, 573F, 647F; 655I, 535N, 573F, 647F; 651F, 589V, 573F, 647F; 655I, 589V, 573F, 647F; 535N, 589V, 573F, 647F; 651F, 655I, 204I, 647F; 651F, 535N, 204I, 647F; 655I, 535N, 204I, 647F; 651F, 589V, 204I, 647F; 655I, 589V, 204I, 647F; 535N, 589V, 204I, 647F; 651F, 573F, 204I, 647F; 655I, 573F, 204I, 647F; 535N, 573F, 204I, 647F; and 589V, 573F, 204I, 647F.

[0143] Embodiment 15 is the recombinant HIV Env protein of embodiment 9, wherein the at least five indicated positions and residues are a combination selected from the group consisting of: 651F, 655I, 535N, 589V, 573F; 651F, 655I, 535N, 589V, 204I; 651F, 655I, 535N, 573F, 204I; 651F, 655I, 589V, 573F, 204I; 651F, 535N, 589V, 573F, 204I; 655I, 535N, 589V, 573F, 204I; 651F, 655I, 535N, 589V, 647F; 651F, 655I, 535N, 573F, 647F; 651F, 655I, 589V, 573F, 647F; 651F, 535N, 589V, 573F, 647F; 655I, 535N, 589V, 573F, 647F; 651F, 655I, 535N, 204I, 647F; 651F, 655I, 589V, 204I, 647F; 651F, 535N, 589V, 204I, 647F; 655I, 535N, 589V, 204I, 647F; 651F, 655I, 573F, 204I, 647F; 651F, 535N, 573F, 204I, 647F; 655I, 535N, 573F, 204I, 647F; 651F, 589V, 573F, 204I, 647F; 655I, 589V, 573F, 204I, 647F; and 535N, 589V, 573F, 204I, 647F.

[0144] Embodiment 16 is the recombinant HIV Env protein of embodiment 10, wherein the at least six indicated positions and residues are a combination selected from the group consisting of: 651F, 655I, 535N, 589V, 573F, 204I; 651F, 655I, 535N, 589V, 573F, 647F; 651F, 655I,

535N, 589V, 204I, 647F; 651F, 655I, 535N, 573F, 204I, 647F; 651F, 655I, 589V, 573F, 204I, 647F; 651F, 535N, 589V, 573F, 204I, 647F; and 655I, 535N, 589V, 573F, 204I, 647F.

[0145] Embodiment 17 is the recombinant HIV Env protein of any of embodiments 1 to 16, further comprising an amino acid substitution by the indicated amino acid residue at at least one of the indicated position selected from the group consisting of:

- (viii) Gln, Glu, Ile, Met, Val, Trp, or Phe, preferably Gln or Glu, at position 588;
- (ix) Lys at position 64 or Arg at position 66 or both Lys at position 64 and Arg at position 66;
- (x) Trp at position 316;
- (xi) Cys at both positions 201 and 433;
- (xii) Pro at position 556 or 558 or at both positions 556 and 558; and
- (xiii) replacement of the loop at amino acid positions 548-568 (HR1-loop) by a loop having 7-10 amino acids, preferably a loop of 8 amino acids, e.g. having a sequence chosen from any one of (SEQ ID NOs: 12-17);
- (xiv) Gly at position 568, or Gly at position 569, or Gly at position 636, or Gly at both positions 568 and 636, or Gly at both positions 569 and 636; and/or
- (xv) Tyr at position 302, or Arg at position 519, or Arg at position 520, or Tyr at position 302 and Arg at position 519, or Tyr at position 302 and Arg at position 520, or Tyr at position 302 and Arg at both positions 519 and 520,

wherein the numbering of the positions is according to the numbering in gp160 of HIV-1 isolate HXB2.

[0146] Embodiment 18 is the recombinant HIV Env protein of any of embodiments 1 to 17, further comprising a mutation in a furin cleavage sequence of the HIV Env protein.

[0147] Embodiment 19 is the recombinant HIV Env protein of embodiment 18, wherein the mutation in the furin cleavage site is a replacement at positions 508-511 by RRRRRR (SEQ ID NO: 10).

[0148] Embodiment 20 is the recombinant HIV Env protein of any of embodiments 1 to 19, being a gp140 or gp160.

[0149] Embodiment 21 is the recombinant HIV Env protein of any of embodiments 1 to 20, wherein the recombinant HIV Env protein has at least one of an improved percentage of trimer formation and an improved trimer yield compared to an HIV Env protein not having the one or

more of the indicated amino acid residues at the indicated positions selected from the group consisting of (i) to (vii).

[0150] Embodiment 22 is the recombinant HIV Env protein of embodiment 21, wherein trimer formation is measured by size exclusion chromatography with multi-angle light scattering (SEC-MALS).

[0151] Embodiment 23 is the recombinant HIV Env protein of any of embodiments 1 to 22, further comprising an amino acid residue chosen from Val, Ile, Phe, Met, Ala, or Leu, preferably Val or Ile, most preferably Val, at position 658.

[0152] Embodiment 24 is the recombinant HIV Env protein of any of embodiments 1 to 23, comprising the combination of amino acids chosen from the group consisting of:

(a) 655I, 589V, 573F, 651F, 588E, 535N, 204I;

(b) 556P, 655I, 535N, 573F, 589V, 204I, 588Q;

(c) 204I, 535N, 556P, 588E, 589V, 651F, 655I;

(d) 535N, 556P, 589V, 651F, 655I; and

(e) 535N, 556P, 588E, 589V, 651F, 655I.

[0153] Embodiment 25 is the recombinant HIV Env protein of any of embodiments 1 to 24, comprising an amino acid sequence that is at least 95%, 96%, 97%, 98%, 99% identical to, or 100% identical to, any one of SEQ ID NOs: 3, 5, 20, 22, 24, 26, 27, 28, 29, 30, 31, or 32, preferably at least 98% identical to any one of SEQ ID NOs: 20, 22, 24, 26, 27, 28, 29, 30, 31, or 32.

[0154] Embodiment 26 is a trimeric complex comprising a noncovalent oligomer of three of the recombinant HIV Env proteins of any of embodiments 1 to 25.

[0155] Embodiment 27 is a particle, e.g. liposome or nanoparticle, e.g. self-assembling nanoparticle, displaying the recombinant HIV Env protein of any one of embodiments 1-25 or the trimeric complex of embodiment 26.

[0156] Embodiment 28 is an isolated nucleic acid molecule encoding a recombinant HIV Env protein of any of embodiments 1 to 25.

[0157] Embodiment 29 is a vector comprising the isolated nucleic acid molecule of embodiment 28 operably linked to a promoter.

[0158] Embodiment 30 is the vector of embodiment 29, which is an adenovirus vector.

[0159] Embodiment 31 is a host cell comprising the isolated nucleic acid molecule of embodiment 28 or the vector of embodiment 29 or 30.

[0160] Embodiment 32 is a method of producing a recombinant HIV Env protein, comprising growing the host cell of embodiment 31 under conditions suitable for production of the recombinant HIV Env protein.

[0161] Embodiment 33 is a method of producing a recombinant HIV Env protein comprising obtaining an expression vector comprising the isolated nucleic acid of embodiment 28 operably linked to a promoter; transfecting a cell with the expression vector; growing the transfected cell under conditions suitable for expression of the recombinant HIV Env protein; and purifying the recombinant HIV Env protein under conditions that permit formation of a stabilized trimeric complex.

[0162] Embodiment 34 is a method of producing a recombinant HIV Env protein according to any one of embodiments 1 to 25, comprising introducing at least one amino acid substitution resulting in the indicated amino acid residue at a position selected from the group consisting of (i)-(vii) into a backbone HIV envelope protein sequence.

[0163] Embodiment 35 is the method according to embodiment 34, wherein a nucleotide sequence encoding the amino acid substitution is introduced into nucleic acid encoding the backbone HIV envelope protein sequence.

[0164] Embodiment 36 is the method of embodiments 34 or 35, wherein the backbone HIV envelope protein sequence is selected from the group consisting of: SEQ ID NO: 2; SEQ ID NO: 3; SEQ ID NO: 4; SEQ ID NO: 5; SEQ ID NO: 6; SEQ ID NO: 7; SEQ ID NO: 8; SEQ ID NO: 9; SEQ ID NO: 6 having a mutation of Glu to Arg at position 166; SEQ ID NO: 6 having Cys at positions 501 and 605 and Pro at position 559 and/or having SEQ ID NO: 10 replacing amino acids 508-511; SEQ ID NO: 6 having a mutation of Glu to Arg at position 166, further having Cys at positions 501 and 605 and Pro at position 559 and/or having SEQ ID NO: 10 replacing amino acids 508-511; SEQ ID NO: 8 having Cys at positions 501 and 605 and Pro at position 559 and/or having SEQ ID NO: 10 replacing amino acids 508-511; SEQ ID NO: 9 having Cys at positions 501 and 605 and Pro at position 559 and/or having SEQ ID NO: 10 replacing amino acids 508-511; and a wild-type HIV Env protein having mutations that result in at least (a), (b) or (c), preferably at least two of (a), (b) and (c), most preferably (a), (b) and (c) of the following: (a) Cys at positions 501 and 506 and Pro at position 559,

(b) having SEQ ID NO: 10 replacing amino acids 508-511, and/or

(c) at least one repair mutation at an amino acid residue that is present at the corresponding position at a frequency of less than 7.5%, preferably less than 2%, of HIV Env sequences in a collection of at least 100, preferably at least 1000, preferably at least 10000, wild-type HIV Env sequences, wherein the repair mutation is a substitution by an amino acid residue that is present at the corresponding position at a frequency of at least 10% of HIV Env sequences in said collection and preferably the repair mutation is a substitution by the amino acid residue that is present at the corresponding position most frequently in said collection.

[0165] Embodiment 37 is a composition comprising the recombinant HIV Env protein of any of embodiments 1 to 25, the trimeric complex of embodiment 26, the particle of embodiment 27, the isolated nucleic acid molecule of embodiment 28, the vector of embodiment 29 or 30, or the host cell of embodiment 31, and a pharmaceutically acceptable carrier.

[0166] Embodiment 38 is a composition of embodiment 37, further comprising an adjuvant.

[0167] Embodiment 39 is a method of producing the composition of embodiment 37, comprising mixing the recombinant HIV Env protein, trimeric complex, particle, isolated nucleic acid, vector, or host cell with one or more pharmaceutically acceptable carriers.

[0168] Embodiment 40 is a method of vaccinating a subject against an HIV infection comprising administering to the subject a composition comprising the recombinant HIV envelope protein of any one of embodiments 1 to 25, the trimeric complex of embodiment 26, the particle of embodiment 27, or the vector of embodiment 29 or 30.

[0169] Embodiment 41 is a method of producing an immune response against an HIV infection in a subject in need thereof, comprising administering to the subject a composition comprising the recombinant HIV envelope protein of any one of embodiments 1 to 25, the trimeric complex of embodiment 26, the particle of embodiment 27, or the vector of embodiment 29 or 30.

[0170] Embodiment 42 is a recombinant HIV Env protein comprising the amino acid sequence of SEQ ID NO: 2, or a sequence that is at least 95% identical thereto.

[0171] Embodiment 43 is a recombinant HIV Env protein comprising the amino acid sequence of SEQ ID NO: 3, or a sequence that is at least 95% identical thereto.

[0172] Embodiment 44 is a recombinant HIV Env protein comprising the amino acid sequence of SEQ ID NO: 4, or a sequence that is at least 95% identical thereto.

[0173] Embodiment 45 is a recombinant HIV Env protein comprising the amino acid sequence of SEQ ID NO: 5, or a sequence that is at least 95% identical thereto.

[0174] Embodiment 46 is an isolated nucleic acid molecule encoding the recombinant HIV Env protein of any of embodiments 42 to 45.

[0175] Embodiment 47 is a vector comprising the isolated nucleic acid molecule of embodiment 46 operably linked to a promoter.

[0176] Embodiment 48 is the vector of embodiment 47, which is an adenovirus vector.

[0177] Embodiment 49 is a host cell comprising the isolated nucleic acid molecule of embodiment 46 or the vector of embodiment 47 or 48.

[0178] Embodiment 50 is a composition comprising the recombinant HIV Env protein of any of embodiments 42 to 45, the isolated nucleic acid molecule of embodiment 46, the vector of embodiment 47 or 48, or the host cell of embodiment 49, and a pharmaceutically acceptable carrier.

[0179] Embodiment 51 is a method to improve the trimer percentage and/or trimer yield (representing folding and stability) of a parent HIV Env protein, the method comprising repairing the amino acid sequence of the parent HIV Env protein by introducing at least one repair mutation, preferably at least 3 repair mutations in the parent HIV Env protein, wherein a repair mutation is an amino acid substitution at an amino acid residue that is found at the corresponding position at a frequency of less than 7.5%, preferably less than 2%, of HIV Env sequences in a collection of at least 100, preferably at least 500, preferably at least 1000, preferably at least 10000, wild-type HIV Env sequences, wherein the substitution is by an amino acid residue that is found at the corresponding position at a frequency of at least 10% of HIV Env sequences in said collection and preferably the substitution is by the amino acid residue that is found at the corresponding position most frequently in said collection.

[0180] Embodiment 52 is the method of embodiment 51, wherein at least 50%, preferably at least 80%, of the amino acid residues in the parent HIV Env protein that are found at corresponding positions at a frequency of less than 7.5% of HIV Env sequences in said collection are repaired.

[0181] Embodiment 53 is the method of embodiment 51, wherein at least 50%, preferably at least 80%, of the amino acid residues in the parent HIV Env protein that are found at

corresponding positions at a frequency of less than 2% of HIV Env sequences in said collection are repaired.

[0182] Embodiment 54 is the method of any one of embodiments 51 to 53, wherein the parent HIV Env protein is from clade C.

[0183] Embodiment 55 is the method of any one of embodiments 51 to 54, wherein the parent HIV Env protein is a wild-type HIV Env protein.

[0184] Embodiment 56 is the method of any one of embodiments 51 to 54, wherein the parent HIV Env protein comprises one or more of the following:

- (a) Cys at positions 501 and 506 and Pro at position 559;
- (b) a mutation in a furin cleavage sequence of the HIV Env protein, e.g. having SEQ ID NO: 10 replacing amino acids 508-511;
- (c) Phe at position 651;
- (d) Ile at position 655;
- (e) Asn at position 535;
- (f) Val at position 589;
- (g) Phe at position 573;
- (h) Ile at position 204;
- (i) Phe at position 647;
- (j) Val at position 658;
- (k) Gln or Glu at position 588; and/or
- (l) Pro at position 556, 558, or 556 and 558.

[0185] Embodiment 57 is a recombinant HIV Env protein obtainable by the method of any of embodiments 51 to 56.

EXAMPLES

[0186] Example 1: Generation of HIV Envelope Clade C and Clade B Consensus Sequence

[0187] HIV Envelope Clade C Consensus Sequence

[0188] An HIV clade C envelope (Env) protein consensus sequence was developed as the backbone sequence for studying the effects of various mutations on trimer formation of the HIV Env proteins. A sequence alignment of 3,434 envelope protein sequences from known HIV viral

isolates was downloaded from the Los Alamos Database (<http://www.hiv.lanl.gov/content/index>). From the 3,434 sequences, 1,252 sequences of clade C only were selected to generate the HIV clade C Env protein consensus sequence. At positions for which a consensus residue could not be clearly identified based on the alignment, the consensus sequence was used to identify the closest wild-type sequences by a BLAST search. The consensus residue at these positions was then selected as the amino acid in the closest wild-type sequences identified from the BLAST search. The HIV Env clade C consensus sequence is shown in SEQ ID NO: 2. The two sequences with the highest homology to SEQ ID NO: 2 using BLAST were the sequences with Genbank numbers ADM30337.1 and ADM30340.1, both having 90% sequence identity to SEQ ID NO: 2.

[0189] The HIV Env clade C consensus sequence was further modified by introducing the so-called SOSIP mutations, which include cysteine residues at positions 501 and 605 and a proline residue at position 559, as well as optimizing the furin cleavage site by replacing the furin site at residues 508-511 with 6 arginine residues. Further, Val at position 295 was mutated into an Asn (V295N), to create an N-linked glycosylation site present in the majority of HIV strains and that can improve binding to certain antibodies used in some experiments.

Additionally, the C-terminus was truncated at residue 664, resulting in a sequence encoding a soluble HIV gp140 protein. All positions of substitution/modification described above are relative to the numbering in gp160 of HIV-1 isolate HXB2. The resulting HIV gp140 sequence, referred to as "ConC_SOSIP," is shown in (SEQ ID NO: 3). The ConC_SOSIP sequence was used as the backbone or parent HIV envelope sequence into which additional mutations, e.g., single and double amino acid substitutions, were introduced to produce recombinant HIV Env proteins according to embodiments of the invention.

[0190] HIV Envelope Clade B Consensus Sequence

[0191] An HIV Env clade B consensus sequence was generated using a similar procedure as that described above for generating the HIV Env clade C consensus sequence. The clade B consensus sequence was generated using 1,708 clade B envelope protein sequences from known clade B viral isolates. The HIV Env clade B consensus sequence is shown in SEQ ID NO: 4.

[0192] The HIV Env clade B consensus sequence was further modified by introducing the so-called SOSIP mutations, optimizing the furin cleavage site by replacing the furin site with 6 arginine residues, and truncating the C-terminus at residue 664, as described above, resulting in a

sequence encoding a soluble HIV gp140 clade B consensus sequence. The resulting HIV gp140 Env protein sequence, referred to as “ConB_SOSIP” is shown in (SEQ ID NO: 5).

[0193] It was surprisingly found that the consensus-based molecules had improved expression levels over molecules based on natural isolates, and moreover already had improved trimerization levels. Hence, the molecules having SEQ ID NOs: 2-5 already have surprisingly advantageous properties.

[0194] Example 2: Expression and Purification of Recombinant HIV Env Protein

[0195] Recombinant HIV Env proteins were expressed and purified as soluble gp140 proteins. Single mutations (amino acid substitutions) and combinations thereof (e.g., double and triple mutations) were introduced into the ConC_SOSIP backbone consensus sequence to generate a series of recombinant HIV Env protein variants.

[0196] Generation and Expression of HIV gp140 Env Constructs and Variants

[0197] DNA encoding the HIV clade C Env consensus sequence ConC_SOSIP shown in SEQ ID NO: 3 was synthesized and codon-optimized at GenScript (Piscataway, NJ 08854) or Gene Art (Life Technologies, Carlsbad, CA). The codon-optimized sequence was then cloned into the vector pcDNA2004 to generate an HIV clade C gp140 Env construct, which was used as the backbone HIV envelope sequence for introducing further mutations. Mutations were introduced into the ConC_SOSIP backbone sequence by site directed mutagenesis and polymerase chain reaction (PCR) performed on the pcDNA2004 HIV clade C gp140 Env construct. HEK-Expi293F cells or HEK293F cells were transiently transfected with 90% of the pcDNA2004 vector encoding the ConC_SOSIP sequence or variant thereof and 10% of a pcDNA2004 vector encoding the furin protease (furin-pCDNA2004) according to the manufacturer's instructions. The transfected cells were cultured for 5 days at 37°C and 10% CO₂. Culture supernatants were spun for 10 minutes at 1250 x g. The spun supernatant was subsequently sterile filtered using a 0.22 µm vacuum filter and stored at 4°C until further use. For expressions in 96-well format the cells were cultured for 3 days at 37°C and 10% CO₂. 4 uL of Optimem (culture medium) was mixed with 4 uL 100 ng/uL DNA and 8 uL Expi293F mix (54 uL/mL Optimem) as added and incubated for 20 minutes. Subsequently 200 uL/well Expi293F cells were added at 2.5 x 10E6 cells/mL. The culture supernatant was harvested and spun for 5

minutes at 300 g to remove cells and cellular debris. The spun supernatant was subsequently sterile filtered using a 0.22 µm vacuum filter and stored at 4°C until further use.

[0198] Purification of HIV gp140 Env Protein

[0199] HIV gp140 Env protein expressed from the pcDNA2004 vector was purified according to a two-step purification protocol using a *Galantus nivalis*-lectin column (Vectorlabs, AL-1243) for the initial purification, and a Superdex200 Increase column (GE) in a subsequent step to remove residual contaminants. For the initial step using the Galantus nivalis-lectin column, culture supernatant was diluted with buffer (40 mM Tris, 500 mM NaCl pH 7.5) and passed over a 4 mL CV Tricorn 10-50 Lectin Agarose Column at a rate of 4 mL per minute. Subsequently, the column was washed with four column volumes buffer (40 mM Tris, 500 mM NaCl pH 7.5) and eluted with four column volumes of 40 mM Tris, 500 mM NaCl, and 1 M mannopyranoside pH 7.5 with an upflow of 1.6 mL/min, meaning that the direction of flow has been changed from down to up to increase the rate of elution of envelope protein and decrease the elution volume. The eluate was concentrated using a spin concentrator (50K, Amicon Ultra, Millipore).

[0200] The HIV gp140 Env protein was further purified on a Superdex200 column using 50 mM Tris, 150 mM NaCl pH 7.4 as running buffer. The second peak that eluted from the column contained the HIV gp140 Env protein. The fractions containing this peak were pooled, and the identity of the peak confirmed as HIV gp140 Env protein using Western blot and SDS-PAGE, and/or SEC-MALS analysis. The concentration of the purified HIV gp140 Env protein was determined by measuring the optical density at 280 nm, and the purified HIV gp140 Env protein was stored at 4°C until further use.

[0201] SDS-PAGE and Western Blotting Analysis

[0202] Cell culture supernatants containing expressed HIV gp140 Env protein and purified HIV gp140 Env protein samples were analyzed on 4-12% (w/v) Bis-Tris NuPAGE gels, 1X MOPS (Life Technologies) under reducing or non-reducing conditions, and blotted using the iBlot technology (Life Technologies). All procedures were performed according to the manufacturer's instructions. For purity analysis, the gels were stained with Krypton Infrared Protein Stain (Thermo Scientific) or SYPRO Rubi protein stain (Bio-Rad). For Western blotting analysis, membranes were probed with an anti-6x-Histidine-Tag antibody (anti-His-HRP). The

gels and the blot membranes were scanned on an Odyssey instrument (Li-Cor), and images were analyzed using Odyssey 3.0 software (Li-Cor).

[0203] Imaging of HIV Env Trimer Formation by Negative Stain Electron Microscopy

Negative stain electron microscopy (NS-EM) was used to image trimers of the envelope protein having the ConC_SOSIP backbone sequence, which were purified using *Galanthus nivalis* lectin followed by size exclusion chromatography, and was performed as described in Julien et al. 2015 (*Proc. Natl. Acad. Sci.* (2015) 112(38) 11947-52). Trimer samples were diluted to between 0.01-0.5 mg/mL in Tris-buffered saline (TBS), pH 7.4 and adhered onto a carbon-coated 200 Cu mesh grid (EMS CF200-Cu) that had been glow discharged in air 2×10^{-1} mbar, 25 mA, 30 seconds, just before use. Subsequently, a 3 μ L drop of the diluted trimer sample was applied to the grid for 1 min followed by blotting with filter paper (Whatman no. 1 or 4). The grids were dried for one minute, then stained with 3 μ L of 2.3% uranyl acetate (UAc) for 60 seconds. Data were collected using an FEI Tecnai F20 electron microscope operating at 120 keV, with a magnification of 25,000x that resulted in a pixel size of 4.68 Å at the specimen plane. Images were acquired with a Gatan BM ultrascan.

Almost all particles in the images (of this trimer-enriched material) were well-formed closed trimers (data not shown).

[0204] Example 3: Screening of Recombinant HIV gp140 Env Variants for Trimer Yield and Percentage of Trimer Formation

[0205] The recombinant HIV Env protein variants generated in Example 2 were screened for trimer formation to identify those mutations that improved the percentage of trimer formed and/or improved trimer yields relative to the ConC_SOSIP backbone sequence. High throughput screening of trimer percentage and trimer yields was conducted using an AlphaLISA assay to evaluate the binding of a panel of broadly neutralizing HIV antibodies (bNAbs) and non-bNAbs to the recombinant HIV Env proteins. The results of the AlphaLISA assay were confirmed by size exclusion chromatography and multi-angle light scattering (SEC-MALS).

[0206] AlphaLISA® Assay Analysis

[0207] Total expression of the HIV gp140 Env protein and the total amount of correctly folded native trimer of over 200 HIV gp140 variants with single amino acid substitutions introduced into the ConC_SOSIP sequence generated as described in Example 2 were measured

in cell culture supernatant by AlphaLISA assay. HIV gp140 variants containing double and triple mutations were also tested. The HIV Env protein having the ConC_SOSIP sequence without any additional mutations was tested for comparison.

[0208] The following monoclonal antibodies (mAbs) were inter alia used for analysis: mAb PGT145, mAb PGDM1400, mAb PG16, mAb PGT151, mAb 35O22, mAb PGT128, mAb PG9, mAb F105, mAb B6, mAb 447-52d, mAb 14e, and mAb 17b. MAbs 447-52D (AB014), PG9 (AB015), and PG16 (AB016) were purchased from Polymun Scientific Immunbiologische Forschung GmbH (Klosterneuburg, Austria). The non-neutralizing antibody b6 was obtained from Dennis R. Burton (The Scripps Research Institute, La Jolla, CA), and the non-neutralizing antibody 14e was obtained from James E. Robinson (Tulane University, New Orleans, LA). For mAbs PGT145 (PDB: 3U1S), PGDM1400 (PDB: 4RQQ), PGT151 (PDB: 4NUG), 35O22 (PDB: 4TVP), F105 (PDB: 1U6A), PGT128 (PDB: 3TYG), and 17b (PDB: 4RQS) nucleic acids encoding the published sequences were cloned into an expression vector and produced for evaluation of the HIV Env proteins. With the exception of mAbs F105, B6, 447-52d, 14e, and 17b, the antibodies used for analysis are broadly neutralizing antibodies (bNAbs). bNAbs are capable of neutralizing multiple HIV viral strains. Of the bNAbs, PGT145, PGDM1400, and PG16 are apex binders and are trimer specific. PGT151 is also trimer specific, but binds at the interface of two protomers of gp120 and gp41, and is cleavage dependent. Binding of non-bNAbs is indicative of incorrect folding or an open trimer conformation.

[0209] Protein folding was also tested by measuring the binding of soluble HIV gp140 Env protein variants to an antibody (mAb 17b) known to bind the co-receptor binding site of the HIV envelope protein, which is exposed only after binding of CD4 (data not shown). In particular, soluble receptor CD4 (sCD4) was used in combination with mAb 17 to evaluate CD4-induced conformational change. Binding of mAb 17b to the HIV gp140 Env protein variant without prior CD4 binding to the envelope protein is an indication of partially unfolded or pre-triggered envelope protein (i.e., an unstable Env that adopts the “open” conformation in the absence of CD4 binding).

[0210] For the AlphaLISA assay, HIV gp140 Env constructs in the pcDNA2004 vector containing a linker followed by a sortase A tag followed by a Flag- tag followed by a flexible (G₄S)₇ linker and ending with a His-tag, were prepared (the sequence of the tag, which was placed at the C-terminus of the HIV Env protein, is provided in SEQ ID NO: 19). The HIV

gp140 Env constructs were expressed in HEK-Expi293 cells, which were cultured for three days in 96 well plates (200 μ L/well). Crude supernatants were diluted 120 times in AlphaLISA buffer (PBS + 0.05% Tween-20 + 0.5 mg/mL BSA). For mAb 17b based assays, supernatants were diluted 12 times. Then, 10 μ L of each dilution were transferred to a 96-well plate and mixed with 40 μ L acceptor beads, donor beads, and one of the above listed mAbs. The donor beads were conjugated to ProtA (Cat#: AS102M, Lot#1831829, Perkin Elmer), which binds to the mAb. The acceptor beads were conjugated to an anti-His antibody (Cat#: AL128M, Perkin Elmer), which binds to the His-tag of the construct. For quantification of the total protein yield, including all forms of the envelope protein, a combination of Nickel-conjugated donor beads (Cat#: AS101M, Perkin Elmer) for detection of the His-tag together with anti-Flag antibody-conjugated acceptor beads (Cat#: AL112R, Perkin Elmer) for detection of the Flag tag were used. For the tests using mAb 17b in combination with sCD4-His, a combination of ProtA donor beads and anti-Flag acceptor beads were used (data not shown). One sample was mixed with donor and acceptor beads to detect trimer formation, and a second sample of the same Env variant was mixed with nickel-conjugated donor beads and anti-Flag conjugated acceptor bead to measure the total amount of protein expressed (i.e., total protein yield).

[0211] The mixture of the supernatant containing the expressed HIV gp140 Env protein, the mAb, donor beads, and acceptor beads was incubated at room temperature for 2 hours without shaking. Subsequently, the chemiluminescent signal was measured with a Synergy NEO plate reader instrument (BioTek). The average background signal attributed to mock transfected cells was subtracted from the AlphaLISA counts measured for each the HIV gp140 Env variants. Then, the whole data set was divided by signal measured for the HIV Env protein having the ConC_SOSIP backbone sequence signal to normalize the signal for each of the HIV gp140 Env variants tested to the backbone. Binding data for each of the HIV gp140 Env variants to the trimer specific mAb PGT145 was used to determine the percentage of trimer formation and trimer yield for each of the variants. Binding to the other mAbs was used to evaluate the general binding pattern of the HIV Env variants to bNAbs and non-bNAbs (not shown).

[0212] The percentage of trimer formation for each of the HIV Env variants was calculated by dividing the normalized chemiluminescent signal obtained from sample mixture of HIV Env variant, the mAb PGT145, ProtA-conjugated donor beads, and anti-His-conjugated acceptor

beads, by the normalized chemiluminescent signal obtained from the sample mixture of the HIV Env variant, anti-His-conjugated donor beads and anti-Flag-conjugated acceptor beads.

[0213] Trimer yield for each of the HIV Env variants was determined relative to the trimer yield for the HIV Env protein having ConC_SOSIP backbone sequence without any additional mutations. The normalized chemiluminescent signal obtained from the binding of mAb PGT145 to the ConC_SOSIP envelope protein was set to 1, and the normalized chemiluminescent signal obtained from the binding of mAb PGT145 to each of the HIV gp140 proteins was normalized to this value.

[0214] Results of AlphaLISA Assay Analysis- Trimer Percentage and Trimer Yields

[0215] The percentage of trimer formation as determined by the AlphaLISA assay for several single, double, and triple amino acid substitutions from the list of (i)-(vii) in Table 1 above in the ConC_SOSIP backbone sequence is shown in FIG. 2A. Of the about 200 HIV gp140 Env variants containing single amino acid substitutions that were tested, seven positions of substitution were identified for which the percentage of trimer formed increased by at least 25% relative to the percentage of trimer formed for the ConC_SOSIP backbone sequence without any additional amino acid substitutions.

[0216] The results shown in FIG. 2A demonstrate that the seven preferred positions of substitution for which a significant increase in the percentage of trimer formation was observed include N651, K655, I535, D589, I573, A204, and E647 according to the numbering in gp160 of HIV-1 isolate HXB2. In particular, the single amino acid substitutions that resulted in the most improved percentage of trimer formation included N651F, K655I(F/W) (although there was also one experiment in which K655F did not appear to result in improvement), I535N, D589V(A), I573F, A204I, E647F. Some mutations that were tested in combination with several of these mutations, included K588Q/E, I556P and A558P, and these further improved the trimer percentage of mutants with preferred amino acids at positions of the invention ((i)-(vii) of Table 1) in this experiment.

[0217] All double substitutions tested in this experiment had a higher percentage of trimer formation than the corresponding single substitutions, and all triple substitutions tested had a higher percentage of trimer formation than the corresponding single and double mutations (FIG. 2A). These unpredictable and surprising results indicate that these mutations could display a form of synergy in these experiments with respect to trimerization of the envelope protein.

[0218] In addition to improved percentage of trimer formation, an increased trimer yield is also desirable. Therefore, the trimer yield of HIV gp140 variants containing single, double, and triple mutations in the ConC_SOSIP backbone sequence was also determined by the AlphaLISA assay. The results are shown in FIG. 2B. Most HIV gp140 variants containing single mutations (exceptions were I535N, D589A and D589I), had a higher trimer yield than the ConC_SOSIP envelope protein. However, the more accurate SEC-MALS analysis of the I535N mutant, as described below, showed an increase in trimer yield. Moreover, additional mutations in combination with I535N, such as D589V, resulted in the same trimer yield observed for the envelope protein having that particular additional substitution in the absence of the I535N mutation. The trimer yield of the variants with double mutations was also increased where each of the single mutation variants had a higher trimer yield than the ConC_SOSIP envelope protein (FIG. 2B).

[0219] The percentage of trimer formation for HIV gp140 variants with double mutations in the ConC_SOSIP backbone that were previously described in the literature was also tested, including the E64K, T316W double substitution described by (De Taeye et al., *supra*), and the disulfide double substitution I204C, A433C described by (Kwon et al., *supra*). The E64K, T316W double substitution resulted in a lower percentage of trimer formation than the ConC_SOSIP envelope protein, i.e., 15% (data not shown). Although the disulfide double substitution I204C, A433C increased the trimer percentage to 43% (data not shown), the novel double substitutions described herein, such as I535N/K588E, K588Q/D589V, K655I/K588E, I535N/D589V, I535N/E647F, D589V/K655I, and I535N/K655I (FIG. 2A) resulted in an even greater percentage of trimer formation in the AlphaLISA experiment.

[0220] Additional mutations (proline at residues 558 and/or 556) were also introduced into the ConC_SOSIP backbone, and the percentage of trimer formation and trimer yield measured for these HIV gp140 Env proteins. Both the single substitutions of Pro at position 558 or 556, and the double substitution of proline at both positions 556 and 558 in addition to the SOSIP mutations already contained in the ConC_SOSIP backbone (i.e., Cys at positions 501 and 605, and Pro at position 559) increased the percentage of trimer formation and trimer yield (data not shown). Indeed, introduction of one or more of the novel amino acid stabilizing substitutions of the invention in the ConC_SOSIP backbone further comprising Pro residues at positions 558

and/or 556 further improves the percentage of trimer formation and/or trimer yield (e.g. Fig. 2A, e.g. A558P/I535N, K655I/L556P, and several triple mutants including the A558P mutation).

[0221] Binding data of the HIV gp140 Env variants to the other bNAbs and non-bNAbs demonstrated that most of the single, double and triple mutations tested which increased trimer yield and the percentage of trimer formation, such as those listed in FIGS. 2A and 2B, also had increased binding to bNAbs, and the same or decreased binding to non-bNAbs relative to the amount of binding observed to the bNAbs and non-bNAbs for the HIV envelope protein having the ConC_SOSIP backbone sequence (data not shown). For vaccine development, increased binding to bNAbs and reduced binding to non-bNAbs is preferred. The data thus demonstrates that the HIV envelope proteins comprising the amino acid substitutions at positions (i)-(vii) indicated in Table 1 above have desirable properties with respect to binding patterns to broadly neutralizing and non-broadly neutralizing antibodies.

[0222] SEC-MALS Analysis

[0223] SEC-MALS analysis was also used to verify the trimer yield and percentage of trimer formation for the HIV gp140 variants screened using the AlphaLISA assay. The HIV gp140 variants were expressed in 30 mL scale cultures and purified by applying the cell free supernatants on 200 µl *Galanthus nivalis* lectin beads (Vectorlab Cat# AL-1243) in Polyrep gravity flow columns (Biorad Cat# 731-1550). The beads were washed with 2 ml binding buffer (40 mM Tris, 500 mM NaCl pH 7.4). The proteins were eluted using 250-500 µl of 40 mM Tris, 500 mM NaCl, 1 M mannopyranoside pH 7.4. A high-performance liquid chromatography system (Agilent Technologies) and MiniDAWN TREOS instrument (Wyatt) coupled to an Optilab T-rEX Refractive Index Detector (Wyatt) was used for performing the SEC-MALS experiment. In total, either 100 µl of lectin elution or approximately 30 µg of protein was applied to a TSK-Gel G3000SWxl column (Tosoh Bioscience) equilibrated in running buffer (150 mM sodium phosphate, 50 mM NaCl, pH 7.0) at 1 mL/min. The data were analyzed using the Astra 6 software package, and molecular weight calculations were derived from the refractive index signal.

[0224] The SEC-MALS chromatograms of the ConC_SOSIP envelope protein and the HIV gp140 variants containing single mutations are shown in FIG. 3. In general, the results obtained from the SEC-MALS analysis were comparable to and consistent with the results obtained from the AlphaLISA analysis. The chromatogram of the ConC_SOSIP envelope protein has four

major peaks, with the second peak that eluted at about 7.3 minutes being the trimer peak. The ConC_SOSIP envelope protein was determined to be about 27% trimeric. The formation of aggregates and monomers indicates that there is some misfolding and instability associated with HIV gp140 Env protein having the ConC_SOSIP consensus sequence. As demonstrated by the chromatograms shown in FIG. 3, all single substitutions resulted in a relatively higher trimer peak as compared to the trimer peak for the ConC_SOSIP envelope protein, indicating that trimer yield was increased for each of the HIV gp140 variants.

[0225] Taken together, the results demonstrate that the amino acid substitutions identified in (i)-(vii) of Table 1 herein provide recombinant HIV Env proteins with improved percentage of trimer formation and/or improved trimer yield. In particular, HIV Env protein variants having multiple substitutions at the identified positions of (i)-(vii) of Table 1, such as combinations of two or more of the identified mutations typically exhibited even more improved trimer yield and/or percentage of trimer formation over HIV Env protein variants having only a single mutation, which shows a possible synergistic effect of combinations mutations (i)-(vii) of Table 1. To the best of the knowledge of the inventors, none of these combinations of amino acid substitutions have been reported in naturally occurring HIV envelope protein sequences, and all combinations (between (i)-(vii)) are thus believed to be novel combinations of trimer stabilizing mutations. HIV envelope proteins having an increased percentage of trimer formation, such as the recombinant HIV envelope proteins of the invention, are advantageous from a manufacturing perspective, such as for vaccines, because less purification and removal of the envelope protein present in the preparation in the undesired non-native conformations will be required. Also, an increased total expression yield of the trimer is advantageous for manufacturing a vaccine product.

[0226] Example 4: Stability of Trimeric HIV Envelope proteins

[0227] The thermal stability of recombinant HIV Env proteins according to embodiments of the invention was tested by AlphaLISA and differential scanning calorimetry (DSC).

[0228] Thermal Stability Measurements Using AlphaLISA

[0229] Heat stability was tested by measuring the loss of intact trimer upon heat treatment based on binding to the trimer-specific mAb PGT145. Crude supernatant (20 µl) was heated at

60°C for 1 hour. The samples were then centrifuged at maximum speed for five minutes to remove aggregates. The AlphaLISA assay was performed as described above in Example 3.

[0230] The results are shown in FIG. 4, and the data is reported as the percentage of trimers left intact after the heat treatment. From the results, it can be seen that most of the single mutant recombinant HIV gp140 Env proteins of the invention tested had higher heat stability than the ConC_SOSIP envelope protein. The HIV envelope proteins having the trimer stabilizing double and triple substitutions identified herein that were tested were also found to have higher heat stability than the ConC_SOSIP envelope protein.

[0231] Thermal Stability Measurements Using DSC

[0232] The melting temperature (T_m) of the HIV gp140 Env variants was determined by DSC using a MicroCal capillary DSC system. Each measurement was performed with a start temperature of 20°C and a final temperature of 110°C at a scan rate of 100°C/hour. A protein sample with a concentration of 0.5 mg/mL (400 μ L) was used for each measurement. The data were analyzed using the Origin J. Software (MicroCal VP-analysis tool).

[0233] The melting temperature (T_m) of the ConC_SOSIP envelope protein measured using DSC was determined to be 69.8°C and the onset temperature of melting was 60.1°C. The T_m measured for the ConC_SOSIP envelope protein was higher than that for the BG505_SOSIP envelope protein (HIV envelope protein of the BG505 viral isolate having the so-called SOSIP mutations), which was reported to have a T_m of 67.0°C (Kwon et al, 2015). This indicates that an HIV envelope protein having the ConC_SOSIP backbone sequence has more favorable properties with respect to heat stability than another known HIV envelope sequence with trimer stabilizing mutations.

[0234] The T_m of the K655I mutant of ConC_SOSIP was measured to be 72.3°C and the onset temperature of melting was 63.7°C, which is even higher than the T_m of ConC_SOSIP envelope protein. The T_m of the A558P, N651F, I535N mutant of ConC_SOSIP was measured to be 77.29°C with an onset temperature of 74.87°C. The DSC results thus confirm the heat stability results determined by the AlphaLISA assay.

[0235] Taken together, the results demonstrate that HIV Env proteins comprising at least one of the amino substitutions described herein typically have higher heat stability than envelope proteins lacking such mutations. The results also demonstrate that all double substitution HIV Env protein variants had higher heat stability than the ConC_SOSIP envelope protein. Triple

substitution HIV Env protein variants were also more stable than the ConC_SOSIP envelope protein.

[0236] Example 5: Recombinant HIV Envelope Protein Variants Based on a Clade B Envelope Protein Consensus Sequence

[0237] Recombinant HIV Env proteins according to embodiments of the invention comprising a single amino acid substitution (I535N, D589V, N651F or K655I) introduced into the clade B consensus sequence ConB_SOSIP (SEQ ID NO: 5) were generated and purified as described in Example 2. The trimer yield and percentage of trimer formation were measured by AlphaLISA assay as described in Example 3.

[0238] The results are shown in FIG. 5A (percentage of trimer formation) and FIG. 5B (trimer yield). The values reported are relative to the value measured for the ConB_SOSIP envelope protein, which was set to 1 for both the percentage of trimer formation and trimer yield. The results show that all of the mutations tested increased the percentage of trimer formation. The trimer yield was about the same or improved relative to the ConB_SOSIP envelope protein for all of the mutations tested.

[0239] These results demonstrate that these mutations also had a stabilizing effect on the envelope protein, e.g., improved trimer yield, improved percentage of trimer formation, etc., when introduced into a different backbone HIV envelope protein sequence, in this case a Clade B derived consensus sequence.

[0240] Example 6: Recombinant HIV Envelope Protein Variants Based on a Synthetic Envelope Protein Sequence

[0241] Recombinant HIV Env proteins according to embodiments of the invention comprising amino acid substitutions introduced into a synthetic HIV envelope protein (named 'DS_sC4_SOSIP_E166R') having the sequence shown in SEQ ID NO: 7 were prepared and purified as described in Example 2. The synthetic HIV envelope protein DS_sC4_SOSIP_E166R has the so-called SOSIP mutations (Cys at residues 501 and 605, and Pro at residue 559), Cys at residues 201 and 433 resulting in the introduction of a disulfide (DS) bond, and Arg at position 166 to stabilize the apex. In addition, the protein is truncated at

position 655. The percentage of trimer formation and trimer yield were measured by AlphaLISA assay as described in Example 3.

[0242] The results are shown in FIG. 6, which compares the percentage of trimer formation for each of the variants tested to the percentage of trimer formation (Fig 6A) and trimer yield (Fig 6B) for the DS_sC4_SOSIP_E166R backbone. A greater percentage of trimer formation was observed for each of the variants tested as compared to the backbone sequence.

[0243] Besides E166R, some other rarely occurring amino acids were changed into more prevalent ones at the corresponding position in a collection of wild-type HIV Env proteins (A114Q, E117K, T375S and I434M), to 'repair' the protein according to a framework explained in more detail in example 12 below and Fig. 12. In this 'repaired' protein, the stabilizing mutations A204I, and K655I improve sC4_SOSIP even further (Fig.13).

[0244] The results of this Example are consistent with those of Example 5 in demonstrating that the mutations described herein also have a stabilizing effect on the envelope protein, e.g., improved percentage of trimer formation, and/or improved trimer yield, etc., when introduced into different backbone HIV envelope protein sequences, in this case a non-consensus, synthetic, Env sequence.

[0245] Example 7: Further combinations of HIV Env mutations

[0246] Recombinant HIV Env proteins according to embodiments of the invention comprising amino acid substitutions introduced in ConC_SOSIP (having the sequence shown in SEQ ID NO: 3) were prepared and purified as described in Example 2. The percentage of trimer formation was measured by AlphaLISA assay as described in Example 3. Subsequently, a smaller selection of combinations (the ones depicted below in *italic* and additionally K655I; I535N,D589V; I535N, K655I; D589V, K655I) were purified using *Galanthus nivalis* lectin and trimer content was analyzed using SEC-MALS as described in Example 3. Stability was measured as described in Example 4.

[0247] The following mutants were prepared for this experiment:

K655I, N651F;

K655I, N651F, E647F;

K655I, N651F, E647F, I535N;

K655I, N651F, I535N;

K655I, I573F;
K655I, D589V, I573F;
K655I, D589V, I573F, N651F;
K655I, D589V, I573F, K588E;
K655I, D589V, I573F, N651F, K588E;
K655I, D589V, I573F, N651F, K588E, I535N;
K655I, D589V, I573F, N651F, K588E, I535N, A204I;
K655I, D589V, I535N, L556P;
K655I, D589V, I573F, N651F, K588E, L556P;
K655I, D589V, A204I;
L556P, N651F;
L556P, N651F, K655I;
L556P, N651F, K655I, I535N;
L556P, N651F, K655I, I535N, I573F;
L556P, N651F, K655I, I535N, I573F, D589V;
L556P, N651F, K655I, I535N, I573F, D589V, A204I;
L556P, N651F, K655I, I535N, I573F, D589V, A204I, K588Q;
L556P, N651F, K655I, I535N, I573F, D589V, A204I, K588Q, E647F;
L556P, N651F, I535N;
L556P, N651F, I535N, I573F;
L556P, N651F, I535N, I573F, D589V;
L556P, N651F, I535N, I573F, D589V, A204I;
L556P, N651F, I535N, I573F, D589V, A204I, K588Q;
L556P, N651F, I535N, I573F, D589V, A204I, K588Q, E647F;
L556P, K655I, I535N;
L556P, K655I, I535N, I573F;
L556P, K655I, I535N, I573F, D589V;
L556P, K655I, I535N, I573F, D589V, A204I;
L556P, K655I, I535N, I573F, D589V, A204I, K588Q;
L556P, K655I, I535N, I573F, D589V, A204I, K588Q, E647F;
L556P, N651F, I535N, I573F, D589V, A204I, K588Q with the SOS mutation removed.

[0248] All tested combinations of substitutions in the ConC_SOSIP backbone showed higher trimer percentage, higher trimer yield and higher trimer stability at 60°C compared to the backbone in AlphaLISA (data not shown). SEC_MALS confirmed improved trimer percentage for all tested mutations in the backbone (data not shown).

[0249] For a set comprising one by one additional mutations up to nine mutations, SEC-MALS showed that by the introduction of each next mutation the ratio trimer/monomer increased, as the height of the monomer peak decreased, while the height of the trimer peak stayed the same in the SEC graph (FIG. 7). Of all the variants tested in SEC-MALS, the variant with L556P, N651F, K655I, I535N, I573F, D589V, A204I, K588Q, E647F substitutions showed the highest trimer percentage (the least gp140 monomers and the least gp120 monomers), the highest total protein yield and one of the higher temperature stabilities. This means that these mutations can be combined without loss of trimer compared to the backbone. In addition, this suggests that, in general, addition of mutations described in (i)-(vii) of Table 1, optionally combined with mutations described in Table 2, results in further improved trimerization.

[0250] A construct with the L556P, N651F, I535N, I573F, D589V, A204I, K588Q mutations wherein the 'SOS mutations' were removed (i.e. the two cysteine residues at positions 501 and 605 were reverted back into the amino acid residues that were originally present in the consensus clade C sequence), was also tested. Even though its temperature stability was lower, the mutant had comparable trimer percentage and yield as its corresponding mutant that did comprise the SOS mutation. The mutant wherein the SOS mutation was removed even had an advantage in that it bound less non-bNAbs than its corresponding SOS-containing counterpart (having the L556P, N651F, I535N, I573F, D589V, A204I, K588Q mutations). This demonstrates that advantageous properties of the instant invention, such as high trimerization percentage, can also be obtained in HIV Env proteins that do not have all the SOSIP mutations.

[0251] One mutant (tested in the ConC_SOSIP backbone), based upon a combination of favorable properties in expression level, trimer formation and binding to broadly neutralizing antibody PGT151, has the following mutations: L556P, N651F, I535N, I573F, D589V, A204I, K588Q.

[0252] In the ConC_SOSIP background, the 9 most successful substitutions were L556P, E647F, N651F, K655I, I535N, D589V, I573F, and K588E in gp41 and A204I in gp120. The combination of all these 9 substitutions led to increased stability, trimer content and trimer yield.

Since addition of L556P in this variant with 9 substitutions had a relatively limited effect on improved trimer percentage, and the E647F substitution in this context appeared to hamper PGT151 binding, these two mutations were not always used in further variants, and a variant with 7 substitutions (named ConC_SOSIP_7mut, sometimes also referred to herein as ‘stabilized ConC_SOSIP’ or ‘ConC_base’; including N651F, K655I, I535N, D589V, I573F, K588E, and A204I) was found to be slightly more stable (increased melting temperature) than the variant with the 9 substitutions indicated above. The complete sequence of this variant (stabilized ConC_SOSIP Env, HIV 160544) is provided in SEQ ID NO: 20.

[0253] At this moment, a particularly preferred mutant [tested in the ConC_SOSIP backbone with the following additional mutations: (a) D279N, A281V, A362Q (increase similarity to transmitted founder viruses, as described by others); (b) Del139-152 (deletion of a variable loop to reduce chance of inducing antibodies to this loop); and (c) V295N (introduction of a glycan site that is present in the majority of HIV strains)], based upon a combination of favorable properties in expression level, trimer formation and binding to a broadly neutralizing antibody, has the following stabilizing mutations of the invention: N651F, K655I, I535N, I573F, D589V, A204I, K588E. The complete sequence of this variant (Stabilized ConC_SOSIP.v3 Env (HIV170654, ConC_SOSIP.v3)) is provided in SEQ ID NO: 28.

[0254] In a further variant, a K658V mutation was added to this construct (see also example 15 below), which further improved the results.

[0255] Example 8: Self-assembling particles displaying stabilized HIV Env protein

[0256] Ferritin and DPS self-assembling particles were prepared that display stabilized Env proteins in a similar fashion as described in (He et al, 2016). In order to do this the gp140 protein was fused to the N-terminus of the particles via a short amino acid linker (e.g. GSG or AAAGS, but other linkers can also be used, see e.g. He et al, 2016) at DNA level and expressed the fusion protein in Expi293F cells. One example of a particle that was prepared in this manner was based on ferritin fused to a ConC_SOSIP (SEQ ID NO: 3) HIV Env protein with the following mutations: I535N, A558P, D589V, K655I. Ferritin particles with this Env protein having an additional V570D mutation, which has been reported to improve trimerization (Kesavardhana et al, 2014), were also prepared, but it was observed that this mutation leads to a strong increase in binding of a non-neutralizing antibody (17b), which is undesired. Env with these five mutations

was also fused to two types of DPS particles, from *Helicobacter pylori* and from *Mycobacterium smegmatis* (see e.g. WO2011/082087 for preparation of DPS particles). Env with these five mutations and in addition the disulfide bridge introducing double mutation I201C-A433C was also fused to ferritin.

[0257] The particles were purified from cell free supernatant with PGDM140 affinity beads and the particles were analyzed using SEC-MALS with a TSKgel G6000PWCL column. SEC-MALS, as well as Native PAGE (3-12%), confirmed that particles with approximately the expected sizes were formed.

[0258] In a similar manner, ferritin and DPS self-assembling nanoparticles displaying HIV Env having a ConC_SOSIP sequence with the following combination of mutations: (L556P, N651F, I535N, I573F, D589V, A204I, K588Q), are also prepared.

[0259] Further liposomes and/or self-assembling nanoparticles displaying other HIV Env variants described herein, e.g. HIV Env having SEQ ID NO: 20, 22, 24, 26, 27, 28, 29, 30, 31, or 32, are also prepared.

Example 9: Recombinant HIV Envelope Protein Variants Based on a Clade A Envelope Protein Sequence

[0260] Recombinant HIV Env proteins according to embodiments of the invention comprising single amino acid substitutions (I535N, D589V, N651F, K655I, I573F, A204I or E647F) were introduced into a wild type clade A HIV envelope protein with the SOSIP modification (named 'BG505_SOSIP') as described in Example 2. The HIV envelope protein BG505_SOSIP has the so-called SOSIP mutations (Cys at residues 501 and 605, and Pro at residue 559), as well as further Cys at residues 201 and 433 resulting in the introduction of a disulfide (DS) bond, and a potential N-glycosylation site on position 332 (T332N mutation). The protein is truncated at position 664. The sequence of BG505_SOSIP is shown in SEQ ID NO: 21.

[0261] The percentage of trimer formation and trimer yield were measured by AlphaLISA assay as described in Example 3. The percentage of trimer formation and trimer yield for each of the variants tested was compared to BG505_SOSIP. A higher percentage of trimer formation was observed for the M535N, D589V, N651F or K655I substitutions as compared to the backbone sequence (e.g. Fig 8A). Combination of e.g. L556P, K655I and M535N showed an even more

increased trimer yield and percentage (e.g. Fig 8A and 8B). Combination of N651F and D589V improved the trimer yield and percentage even more (data not shown). The results of this Example for a clade A virus are consistent with those of examples 10 and 11 (clade C) below and Example 5 (clade B), in which the mutations I535N, D589V, N651F and K655I also showed a stabilizing effect on the envelope protein derived from wild-type strains, e.g., improved percentage of trimer formation, and/or improved trimer yield. Clearly, these mutations of the invention also improve trimerization of HIV Env derived from a wild-type clade A strain.

[0262] At this moment, a particularly preferred mutant (tested in the BG505_SOSIP backbone, based upon a combination of favorable properties in expression level, trimer formation and binding to a broadly neutralizing antibody, is the one having the following mutations: L556P, K655I, M535N, N651F, D589V, (see e.g, Fig. 9, showing a strongly improved trimer formation of such mutant in a SEC-MALS analysis, and Fig. 13, showing a clearly improved binding of broadly neutralizing antibodies of such mutant). The sequence of this stabilized BG505_SOSIP Env (HIV170863) is shown in SEQ ID NO: 22.

[0263] Addition of mutation Q658V provided a small further improvement.

[0264] A further preferred construct contains the L556P, K655I, M535N, N651F, D589V mutations, as well as the 'DS' mutations (Cys at positions 201 and 433 resulting in introduction of a disulfide bond), R588E, and Q658V. The sequence of that variant (BG505_SOSIP.v2 Env, HIV171814) is provided in SEQ ID NO: 29.

Example 10: Recombinant HIV Envelope Protein Variants Based on clade C Wild Type Envelope Protein Sequence

[0265] Recombinant HIV Env proteins according to embodiments of the invention comprising the single amino acid substitution T651F, the double amino acid substitution T651F, M535N introduced into a WT C97ZA_SOSIP Env sequence (SEQ ID NO: 23) with the additional substitution L556P (C97ZA_SOSIP_L556P) were generated and expressed as described in Example 2. The trimer yield and percentage of trimer formation were measured by AlphaLISA assay as described in Example 3.

[0266] The results are shown in FIG. 10A and B. The trimer yield of C97ZA_SOSIP_L556P_T651F_M535N is five times higher than that of the C97ZA_SOSIP backbone.

[0267] The L556P, T651F and M535N substitutions thus gave a large improvement of C97ZA_SOSIP, but binding to bNAbs and trimer percentage for this clade C wild-type derived variant was still much lower than for the ConC_SOSIP backbone. Because a wt Env may be adapted to its host, possibly reducing its general fitness, and thereby the folding may be corrupted, the Env sequence was 'repaired' according to the conceptual framework described below in Example 12 and in FIG 12. A total of 21 residues were changed, to repair the sequence, and three potential N-glycosylation sites (PNGS) were added to fill the so-called "glycan holes" (positions where in at least 50% of the wild-type HIV strains Env protein a potential N-glycosylation site is present). The mutations introduced by following this framework for C97ZA_SOSIP are indicated in Table 3 in the column 'repairing mutations'. Addition of stabilizing mutation K655I disclosed herein increased the trimer percentage and yield even further, as did D589V, A204I and K588E.

[0268] These results demonstrate that the T651F, M535N and K655I, D589V, A204I and K588E mutations described herein also had a stabilizing effect on the envelope protein, e.g., improved trimer yield, improved percentage of trimer formation when introduced into C97ZA_SOSIP (derived from a clade C wild-type strain Env protein) and variants thereof.

[0269] At this moment, a particularly preferred variant (tested in the C97ZA_SOSIP backbone), based upon a combination of favorable properties in expression level, trimer formation and binding to a broadly neutralizing antibody, is the one having the following mutations: Q567K (described by others before); A198T, S243N, K236T, V295N (to fill glycan holes); M34L, T46K, T58A, Q171K, G172V, P179L, L183Q, I192R, N209T, M307I, Q350R, N352H, Y353F, D412N, G429E, V455T, I489V, L491I, G500K, S547G, T578A, T651N (to repair the sequence); V505N, E507T, T663N (added potential N-glycosylation sites at base of molecule); and A204I, M535N, L556P, K588E, D589V, T651F, K655I (stabilizing mutations of invention). Data for this variant are for instance shown in Fig. 13, see in particular 'stabilized and repaired C97ZA' therein), showing a huge increase in broadly neutralizing antibody binding as compared to the original wt C97ZA Env molecule. The sequence of this variant (stabilized and repaired C97ZA_SOSIP Env (HIV170690)) is provided in SEQ ID NO: 24.

[0270] Addition of mutation K658V stabilized this protein even further.

[0271] A further preferred variant includes the 'DS' mutation and K658V, and the sequence of this variant (C97ZA_SOSIP.v2 Env, HIV171810) is provided in SEQ ID NO: 30.

Example 11: Recombinant HIV Envelope Protein Variants Based on another clade C Wild Type Envelope Protein Sequence

[0272] In the Env protein from clade C strain Du422, SOSIP mutations were introduced and two glycan holes were filled at position 295 and 386 by K295N and D386N mutations. In addition, some residues were repaired according to the conceptual framework described in Example 12 and Fig. 12 (V272I, W456R, G466E and F643Y), and stabilizing substitutions L556P, I535N, N651F and D589V were introduced. All additional substitutions resulted in higher trimer yields and trimer percentages (e.g. Fig. 11).

[0273] In a specific tested variant with these four stabilizing mutations (SEQ ID NO: 25), the additional K655I substitution further increased trimer yield and trimer percentage by a factor 1.3 and 1.4 respectively (data not shown).

[0274] At this moment, a particularly preferred Du422_SOSIP Env variant, based upon a combination of favorable properties in expression level, trimer formation and binding to a broadly neutralizing antibody, is the one having the following mutations: L556P, K655I, M535N, N651F, D589V, K588E, I201C, A433C, V272I, W456R, G466E, F643Y, D386N, and K295N. The sequence of this variant (stabilized and repaired Du422_SOSIP Env (HIV170859) is provided in SEQ ID NO: 26. Data for this variant are for instance shown in Fig 13 (see stabilized and repaired Du422 therein), showing a huge increase in broadly neutralizing antibody binding compared to the original wt Du422 Env molecule.

[0275] A further preferred variant additionally comprises the 'DS' mutation and K658V, and the sequence of this variant (Du422_SOSIP.v1 Env, HIV171812) is provided in SEQ ID NO: 31.

Example 12: Repairing and stabilizing various HIV-1 Env sequences

[0276] Because wt sequences from viruses isolated from infected patients may have acquired destabilizing mutations that impede correct folding, wt Env sequences of clade C C97ZA, DU422 and the mosaic sC4 were first repaired.

[0277] To search for non-optimal mutations in wild type sequences an alignment of all HIV-1 Env sequences in the UniProt database and the Los Alamos HIV database (~90.000 sequences) was made and the amino acid distribution was calculated for each amino acid. In general, a number of relatively rarely occurring amino acids in wt Env sequences were substituted into more common amino acids (based upon frequency in the database at the corresponding position) according to the conceptual framework described in FIG 12.

[0278] Furthermore, two additional substitutions Y353F and Q171K at the apex of C97ZA_SOSIP were introduced to possibly improve the binding of apex targeting antibodies, and extra glycan sites were introduced by the substitution of D411N, K236T and V295N because these potential N-glycosylation sites (PNGS) were conserved >50%. Next, stabilizing substitutions described in previous examples were transferred to the repaired sequence.

[0279] The stabilized ConC_SOSIP contains the substitutions A204I, I535N, I573F, K588E, D589V, N651F and K655I (stabilized ConC_SOSIP). The complete sequence of stabilized ConC_SOSIP is provided in SEQ ID NO: 20.

[0280] An overview of some of the variant Env proteins and their mutations is provided in Table 3.

[0281] Table 3. HIV Env protein variants.

Protein	mutations from literature	added PNGS	leader sequence (SEQ ID NO:)	repairing mutations	stabilizing mutations	other mutations	terminus
ConC_SOSIP		V295N	11				664
Stabilized ConC_SOSIP		V295N	11		A204I, I535N, I573F, K588E, D589V, N651F, K655I		664
BG505_SOSIP		T332N	34				664
stabilized BG505_SOSIP		T332N	34		M535N, L556P, D589V, N651F, K655I		664
C97ZA_SOSIP	L535M, Q567K		native				664
repaired C97ZA_SOSIP	L535M, Q567K	A198T, S243N, K236T, V295N	11	M34L, T46K, T58A, Q171K, G172V, P179L, L183Q, I192R, N209T, M307I, Q350R, N352H, Y353F, D412N, G429E, V455T, I489V, L491I, G500K, S547G, T578A, T651N		V505N, E507T, T663N	664

repaired and stabilized C97ZA_SOSIP	Q567K	A198T, S243N, K236T, V295N	11	M34L, T46K, T58A, Q171K, G172V, P179L, L183Q, I192R, N209T, M307I, Q350R, N352H, Y353F, D412N, G429E, V455T, I489V, L491I, G500K, S547G, T578A, T651N	A204I, M535N, L556P, K588E, D589V, T651F, K655I	V505N, E507T, T663N	664
Du422_SOSIP		D386N, K295N	11				664
repaired Du422_SOSIP		D386N, K295N	11	V272I, W456R, G466E, F643Y			664
repaired and stabilized Du422_SOSIP		D386N, K295N	11	V272I, W456R, G466E, F643Y	M535N, L556P, K588E, D589V, N651F, K655I		664
DS_sC4_SOSIP	I201C-A433C	V295N	33				655
repaired DS_sC4_SOSIP	I201C-A433C	V295N	33	A114Q, E117K, E166R, T375S, I434M			655
repaired and stabilized DS_sC4_SOSIP	I201C-A433C	V295N	33	A114Q, E117K, E166R, T375S, I434M	A204I, I535N, L556P, Q588E, D589V, N651F, K655I	delta138-152 (SSNGTYNII HNETYK), delta191 (SEKSSSENS E), delta 463 (GVP)	655

[0282] Table 3. Several of HIV Env protein variants described herein. The column ‘mutations from literature’ describes mutations that were used in these constructs and previously described by others. The column ‘added PNGS’ describes mutations that add a potential N-glycosylation site (at positions where many wild type Env proteins comprise such a site). The column ‘leader sequence’ describes which leader sequence was used for expression if it was not the original (native) leader sequence. The column ‘repairing mutations’ describes the mutations that improve folding and stability (measured as trimer yield and percentage, based on binding to bNAbs) of some of the wild-type Env proteins, as described in Example 12 and Fig. 12. The column ‘stabilizing mutations’ describes mutations of the invention that stabilize the protein and improve trimerization as disclosed herein. The column ‘further mutations’ describes additional mutations made for some constructs. The column ‘terminus’ describes the position of the last amino acid (numbering throughout the table is with respect to HXB2 Env sequence).

[0283] Supernatants of cells transiently transfected with wild-type (wt), repaired, and stabilized Env variants were tested for binding to several trimer-specific broadly neutralizing antibodies directed to the apex. The repair substitutions and especially the stabilizing substitutions had a dramatic impact on trimer content (Figs. 13 and 14), determined with AlphaLISA (Fig. 13) and SEC-MALS (Fig. 14).

[0284] The sequence of a preferred variant of the repaired and stabilized DS_sC4 Env protein (repaired and stabilized DS_sC4_SOSIP Env (HIV170686)) is provided in SEQ ID NO: 27.

[0285] Another preferred variant thereof is provided in SEQ ID NO: 32 (repaired and stabilized sC4_SOSIP.v4 Env).

[0286] Example 13: Stabilizing mutations of the invention function in the absence of the SOSIP mutations

[0287] As shown in previous examples, the 7 mutations (A204I, I535N, I573F, K588E, D589V, N651F and K655I) improved the trimer yield and percentage in the ConC_SOSIP (resulting in 'ConC_base' or 'stabilized ConC_SOSIP' or 'ConC_SOSIP 7mut') (e.g. Figs. 13 and 15).

[0288] This example demonstrates that the different SOSIP mutations (i.e. the 'SOS' mutation: 2 substitutions by Cys residues at positions 501 and 605; and the 'IP mutation': substitution by Pro residue at position 559) contribute to further stabilization, but are not required to obtain benefits from the mutations of the invention.

[0289] The 7 mutations were shown to also improve trimer yield in the so-called ConC_SOS, which does not contain the stabilizing I559P mutation ('IP' mutation), as shown in Fig. 15 (compare ConC_SOS vs ConC_SOS, 7mut). Hence, the 'IP' mutation is not essential for obtaining a benefit from the mutations described herein. Addition of the I559P mutation resulted in a big increase, showing that the 'IP' mutation is beneficial in this construct in addition to the 7 mutations of the invention. The stabilizing IP mutation (I559P) could also be replaced by A558P or L556P, both of these also resulting in a big increase over the variant lacking the I559P mutation.

[0290] Also the ConC_IP, 7 mut, which contains the 7 mutations of the invention described above, but lacks the 'SOS' mutations, still showed a very high trimer yield, demonstrating that

also the 'SOS' mutations are not essential for obtaining benefit from the mutations described herein (e.g. compare ConC_SOSIP vs ConC_IP, 7 mut), in line with observations in example 7. Addition of the 'SOS' mutation does further increase the trimer yield.

[0291] Thus, while Env trimers containing the stabilizing mutations described herein can benefit from further stabilization with the SOSIP mutations, none of the 3 SOSIP mutations is required for obtaining benefits (e.g. improved trimer yield) of the stabilizing mutations described herein.

[0292] Example 14: Methionine substitution at positions 647, 651 or 655 improves trimer quality

[0293] Further to the mutations described in example 2, positions 589, 647, 651 and 655 were individually substituted by a Met residue in a ConC_SOSIP (SEQ ID NO: 3) backbone, and tested for trimerization percentage and yield using methods as described above. It was shown that a Met at positions 647, 651, or 655, like the mutations described in example 2, improved the quality of the trimer (higher trimer percentage and yield, increased bNAbs binding), as can be seen in Fig. 16.

[0294] Thus, apart from substitution by Phe, Ala, or Trp at position 651, substitution by Met at position 651 also improves trimer formation; apart from substitution by Phe, Ile, or Trp at position 655, substitution by Met at position 655 also improves trimer formation; and apart from substitution by Phe, or Ile at position 647, substitution by Met at position 647 also improves trimer formation.

[0295] Example 15: HIV Env protein with trimer stabilizing mutation at position 658.

[0296] Recombinant HIV Env proteins with substitution mutations at position 658 (numbering according to gp160 of HIV-1 isolate HXB2) were prepared, in the ConC_SOSIP (SEQ ID NO: 3) backbone. K658 was mutated into Val, Ile, Phe, Leu, Met, or Ala. In addition, some double mutants were made wherein these mutations were combined with one of the stabilizing mutations described above, K655I. The percentage of trimer formation was determined by the AlphaLISA assay as described in Example 3.

[0297] Results are shown in Fig. 17A and B (trimer percentage, measured in different experiments, hence two panels) and Fig. 17C and D (trimer yield, measured in different

experiments, hence two panels). These results demonstrate that substitution at position 658 by Ile, Phe, Met, Leu, Ala, or Val resulted in improved percentage of trimer formation and improved trimer yield. Substitution with Ile at position 658 resulted in increases that are in about the same range as the K655I mutation (Fig. 17A, C), which was the best performing single mutant from the mutations (i)-(vii) in Table 1 described above (see e.g. Fig 2A). Substitution with Val at position 658 resulted in even higher improvement (Fig. 17A, C).

[0298] The results also demonstrated that substitution at position 658 by Ile or Val could be combined with mutation K655I that was described above, and that this resulted in a further improvement over each of the corresponding single mutants (Fig. 17A, C).

[0299] The K658V mutant was also tested using SEC-MALS. 96-well cultures were grown for three days as was done for the AlphaLISA. Supernatant was directly loaded on a SEC-MALS column. The chromatograms obtained for the mock supernatant (with furin expression) was subtracted from the chromatograms of the supernatant with Env proteins. The trimeric protein eluted from the column between 7 and 8 minutes. The results are shown in Fig. 18, and confirmed that the K658V mutant showed improved trimerization over the background Env protein, and over the K655I mutant Env protein.

[0300] This example demonstrates that substitution of the amino acid at position 658 in HIV Env protein by Val, Ile, Phe, Met, Leu, or Ala results in improved trimer percentage and trimer yield.

[0301] Further experiments to measure trimer formation of variants using AlphaLISA and/or SEC-MALS are performed in HIV Env variants wherein the K658V mutation is present in combination with other mutations from Tables 1 and/or 2 as described herein, as well as in HIV strains from clade A and B. For example, the 658V mutation has already been shown to improve the ConC_SOSIP_{7mut} variant as described above (example 7), as well as BG505_SOSIP with L556P, K655I, M535N, N651F, D589V, K588E (example 9), as well as the repaired and stabilized C97ZA_SOSIP (example 10).

[0302] Based on the results described above, it is expected that the mutation of the amino acid at position 658 into a Valine, Isoleucine, Phenylalanine, Leucine, Methionine or Alanine, preferably into a Valine, residue will improve trimer formation and/or trimer yield in different background HIV Env proteins.

[0303] Example 16. Immunization with stabilized HIV Env proteins

[0304] A rabbit immunization study is conducted with soluble Env protein and with Env proteins coupled to liposomes. The prime is performed with stabilized ConC_SOSIP.v3 (SEQ ID NO: 28), and followed by four boosts, each with another protein, i.e. with 1) repaired and stabilized sC4_SOSIP.v4 (SEQ ID NO: 32); 2) repaired and stabilized C97ZA_SOSIP.v2 (SEQ ID NO: 30); 3) repaired and stabilized Du422_SOSIP.v1 (SEQ ID NO: 31); and 4) stabilized BG505_SOSIP.v2 (SEQ ID NO: 29).

[0305] Serum is isolated after successive immunizations, and analyzed for induced antibodies that particularly bind to the stable, closed, pre-fusion conformation of Env (using ELISA), as well as for induction of bNAbs (using virus neutralization assays).

[0306] The examples above demonstrate that the invention provides a universal approach to optimize the folding and stability of prefusion-closed HIV envelope trimer proteins.

[0307] It is understood that the examples and embodiments described herein are for illustrative purposes only, and that changes could be made to the embodiments described above without departing from the broad inventive concept thereof. It is understood, therefore, that this invention is not limited to the particular embodiments disclosed, but it is intended to cover modifications within the spirit and scope of the invention as defined by the appended claims.

LIST OF SEQUENCES

SEQ ID NO: 1 gp160 of HIV-1 isolate HXB2 (signal sequence in italics; amino acids at positions (i)-(vii) for mutations according to the invention indicated by grey shading)

MRVKEKYQHLLWRWGWRWGTMLLGMLMICSATEKLWVTVYYGVPVWKEATTTLFCASDAKAYDTEVHNWATHACVPTDPNPQEVVLNVNVTENFNMWKNMVEQMHEDIISLWDQSLKPCVKLTPLCVSLKCTDLKNDTNTNSSSGRMIMEKGEIKNCSFNISTSIRGKVQKEYAFFYKLDIIPIDNDTTSYKLTSCNTSVITQACPVSFEPIPIHYCAPAGFAILKCNKTFNGTGPCNTNVSTVQCTHGIRPVVSTQLLNGSLAEEEVVIRSVNFTDNAKTIIVQLNTSVEINCTRPNNNTRKRIRIQRGPGRAFVTIGKIGNMRQAHCNISRAKWNNTLKQIASKLREQFGNNKTIIFKQSSGGDPEIVTHSFNCGGEFFYCNSTQLFNSTWFNSTWSTEGSNTEGSDTITLPCRIKQIINMWQKVGKAMYAPPISGQIRCSSNITGLLLTRDGGNSNNESEIFRPGGGDMRDNRSELYKYKVVKIEPLGVAPTAKRRVVQREKRAVGIGALFLGFLGAAGSTMGAASMTLTVQARQLLSGIVQQQNLLRAIEAQHLLQLTVWGIKQLQARILAVERYLKDDQQLGIWGCSGKLICTTAVPWNASWSNKSLEQIWNHTTMEWDREINNYTSLIHSLEIESONQOEKNEQELLELDKWASLWNWFNITNWLWYIKLFIMIVGGVLGLRIVFAVLSIVNRVRQGYSPLSFQTHLPTPRGPDRPEGIEEEGGERDRDRSIRLVNGSLALIWDLLRSLCLFSYHRLRDLILLIVTRIVELLGRRGWEALKYWWNLLQYWSQELKNSAVSLLNATAIAVAEGTDRVIEVVQACRAIRHIPRIRI RQGLERILL

SEQ ID NO: 2 HIV Env consensus clade C (consensus sequence only, not including any signal sequence, transmembrane domain (664 is last amino acid), SOSIP mutations, and/or furin cleavage site mutations; amino acids at positions (i)-(vii) for mutations according to the invention indicated by grey shading)

NLWVTVYYGVPVWKEAKTTLFCASDAKAYEKEVHNWATHACVPTDPNPQEMVLENVNVTENFNMWKNMVDQMHEDIISLWDQSLKPCVKLTPLCVTLNCTNVNVTNTNNNNMKEEMKNCSFNNTTTEIRDKKQKEYALFYRLDIVPLNENSSEYRLINCNTSTITQACPVSFDPPIPIHYCAPAGYAILKCNKTFNGTGPCNNVSTVQCTHGKIPVSTQLLNGSLAEEEIIRSENLTDNAKTIIVHLNESVEINCTRPNNNTRKSIIRIGPGQTFYATGDIIGDIRQAHCNISEAKWNKTLQRVKKKLKEHFPNKTIKFAPSSGGDLEITTHSFNCRGEFFYCNTSKLFNSTYNNNTSNSTITLPCRIKQIINMWQEVGRAMYAPPIAGNITCKSNITGLLLTRDGGNNNNNTETFRPGGGDMRDNRSELYKYKVVEIKPLGIAPTAKRRVVVEREKRRAVGIGAVFLGFLGAAGSTMGAASITLTVQARQLLSGIVQQSNLLRAIEAQHMLQLTVWGIKQLQARVLAIERYLKDQQLGIWGCSGKLICTTAVPWNSSWSNKSQEDIWDNMTWMQWDREISNYTDTIYRLLEESONQOEKNEKDLLALD

SEQ ID NO: 3 ConC_SOSIP (mature clade C consensus sequence with SOSIP mutations and furin cleavage site (in italics), and C-terminal truncation; amino acids at positions (i)-(vii) for mutations according to the invention indicated by grey shading)

NLWVTVYYGVPVWKEAKTTLFCASDAKAYEKEVHNWATHACVPTDPNPQEMVLENVNVTENFNMWKNMVDQMHEDIISLWDQSLKPCVKLTPLCVTLNCTNVNVTNTNNNNMKEEMKNCSFNNTTTEIRDKKQKEYALFYRLDIVPLNENSSEYRLINCNTSTITQACPVSFDPPIPIHYCAPAGYAILKCNKTFNGTGPCNNVSTVQCTHGKIPVSTQLLNGSLAEEEIIRSENLTDNAKTIIVHLNESVEINCTRPNNNTRKSIIRIGPGQTFYATGDIIGDIRQAHCNISEAKWNKTLQRVKKKLKEHFPNKTIKFAPSSGGDLEITTHSFNCRGEFFYCNTSKLFNSTYNNNTSNSTITLPCRIKQIINMWQEVGRAMYAPPIAGNITCKSNITGLLLTRDGGNNNNNTETFRPGGGDMRDNRSELYKYKVVEIKPLGIAPTCKRRVVERRRRRAVGIGAVFLGFLGAAGSTMGAASITLTVQARQLLSGIVQQSNLLRAPEAQHMLQLTVWGIKQLQARVLAIERYLKDQQLGIWGCSGKLICTTAVPWNSSWSNKSQEDIWDNMTWMQWDREISNYTDTIYRLLEESONQOEKNEKDLLALD

SEQ ID NO: 4 HIV Env consensus clade B (consensus sequence only, not including any signal sequence, transmembrane domain (664 is last amino acid), SOSIP mutations, and/or furin cleavage site mutations; amino acids at positions (i)-(vii) for mutations according to the invention indicated by grey shading)

AEKLWVTVYYGVPVWKEATTTLFCASDAKAYDTEVHNWATHACVPTDPNPQEVVLNVNVTENFNMWKNMVEQMHEDIISLWDQSLKPCVKLTPLCVTLNCTDLNNNTTNNNSSSEKMEKGEIKNCSFNITTSIRDKVQKEYALFYKLDVVPIDNNNTSYRLISCNTSVITQACPVSFEPIPIHYCAPAGFAILKCNKTFNGTGPCNTNVSTVQCTHGIRPVVSTQLLNGSLAEEEVVIRSENFTDNAKTIIVQLNESVEINCTRPNNNTRKSIHIGPGRFYATGDIIGDIRQAHCNISRTKWN

TLKQIVKKLREQFGNKTIVFNQSSGGDPEIVMHSFNCGGEFFYCNTTQLFNSTWNSNGTWNNTTGNNDITITLPCRIKQ
 IINMWQEVGKAMYAPPPIRGQIRCSSNITGLLLTRDGGNNNNNTTETFRPGGGDMRDNRSELYKYKVVKIEPLGVAP
 TKCKRRVVQRRRRRAVGIGAMFLGFLGAAGSTMGAASITLTVQARQLLSGIVQQNNLLRAPEAQHLLQLTVWGI
 KQLQARVLAVERYLKDQQLLGIWGCSGKLICCTAVPWNTSWSNKSLEIWDNMTWMQWEREIDNYTGLIYTLIEESQ
 NQOEKNEQELLELD

SEQ ID NO: 5 ConB_SOSIP (mature clade B consensus sequence with SOSIP mutations and furin cleavage site (in *italics*), and C-terminal truncation; amino acids at positions (i)-(vii) for mutations according to the invention indicated by grey shading)

AEKLWVTVYYGVPVWKEATTTLFCASDAKAYDTEVHNVWATHACVPTDPNPQEVVLENTENFNMWKNMVEQMHE
 IISLWDQSLKPCVKLTPLCVTLNCTDLNNNTTNNNSSSEKMEKGEIKNCSFNITTSIRDKVQKEYALFYKLDVVPID
 NNNTSYRLISCNTSVITQACPKVSFEPPIPIHYCAPAGFAILKCNDKKFNGTGPCTNVSTVQCTHGIRPVVSTQLLLN
 GSLAEVEEVIRSENFTDNAKTIIIVQLNESVEINCTRPNNNTRKSIHIGPGRFYATGDIIGDIRQAHCNISRTKWN
 TLKQIVKKLREQFGNKTIVFNQSSGGDPEIVMHSFNCGGEFFYCNTTQLFNSTWNSNGTWNNTTGNNDITITLPCRIKQ
 IINMWQEVGKAMYAPPPIRGQIRCSSNITGLLLTRDGGNNNNNTTETFRPGGGDMRDNRSELYKYKVVKIEPLGVAP
 TKCKRRVVQRRRRRAVGIGAMFLGFLGAAGSTMGAASITLTVQARQLLSGIVQQNNLLRAPEAQHLLQLTVWGI
 KQLQARVLAVERYLKDQQLLGIWGCSGKLICCTAVPWNTSWSNKSLEIWDNMTWMQWEREIDNYTGLIYTLIEESQ
 NQOEKNEQELLELD

SEQ ID NO: 6 synthetic HIV envelope protein Mos2S Env C4 fragment; amino acids at positions (i)-(vii) for mutations according to the invention indicated by grey shading)

MGNLWVTVYYGVPVWKAATTTLFCASDAKAYEKEVHNVWATHACVPTDPNPQEIIVLGNVTENFNMWKNMVDQMHE
 IISLWDASLEPCVKLTPLCVTLNCRNVRNVSSNGTYNIIHNETYKEMKNCSFNATTVVEDRKQKVHALFYRLDIVPL
 DENNSSEKSSSENSSEYYRLINCNTSAITQACPKVSFDPIPIHYCAPAGYAILKCNNKTFNGTGPCNNVSTVQCTHGI
 KPVVSTQLLNLSLAEEEEIIIRSENLTNNAKTIIIVHLNETVNITCTRPNNNTRKSIIRIGPGQTFYATGDIIGDIRQA
 HCNLSRDGWNKTQGVKKKLAEHFPNKTIKFAPHSGGDLEITHTFNCRGEFFYCNTSNLFNENIERNDIIITLPC
 RIKQIINMWQEVGRAIYAPPIAGNITCRSNITGLLLTRDGGSNNGVPNDTETFRPGGGDMRNNNRSELYKYKVVEVK
 PLGVAPTEAKRRVVEREKRAVGIGAVFLGILGAAGSTMGAASITLTVQARQLLSGIVQQSNLLRAIEAQHMLQLT
 VWGIKQLQTRVLAIERYLQDQQLLGLWGCSGKLICTTAVPWNTSWSNKSQTDIWDNMTWMQWDKEIGNYTGEIYRL
 EESQNNQOEK

SEQ ID NO: 7 (DS_sC4_SOSIP_E166R sequence; amino acids at positions (i)-(vii) for mutations according to the invention indicated by grey shading)

MGNLWVTVYYGVPVWKAATTTLFCASDAKAYEKEVHNVWATHACVPTDPNPQEIIVLGNVTENFNMWKNMVDQMHE
 IISLWDASLEPCVKLTPLCVTLNCRNVRNVSSNGTYNIIHNETYKEMKNCSFNATTVVRDRKQKVHALFYRLDIVPL
 DENNSSEKSSSENSSEYYRLINCNTSACTQACPKVSFDPIPIHYCAPAGYAILKCNNKTFNGTGPCNNVSTVQCTHGI
 KPVVSTQLLNLSLAEEEEIIIRSENLTNNAKTIIIVHLNETVNINCTRPNNNTRKSIIRIGPGQTFYATGDIIGDIRQA
 HCNLSRDGWNKTQGVKKKLAEHFPNKTIKFAPHSGGDLEITHTFNCRGEFFYCNTSNLFNENIERNDIIITLPC
 RIKQIINMWQEVGRCIYAPPIAGNITCRSNITGLLLTRDGGSNNGVPNDTETFRPGGGDMRNNNRSELYKYKVVEVK
 PLGVAPTECKRRVVERRRRRAVGIGAVFLGILGAAGSTMGAASITLTVQARQLLSGIVQQSNLLRAPEAQHMLQ
 LTVWGIKQLQTRVLAIERYLQDQQLLGLWGCSGKLICCTAVPWNTSWSNKSQTDIWDNMTWMQWDKEIGNYTGEIYR
 LLEESQNNQOEK

SEQ ID NO: 8 (Mos1.Env, mosaic HIV envelope protein sequence; amino acids at positions (i)-(vii) for mutations according to the invention indicated by grey shading)

AGKLWVTVYYGVPVWKEATTTLFCASDAKAYDTEVHNVWATHACVPTDPNPQEVVLENTENFNMWKNMVEQMHE
 IISLWDQSLKPCVKLTPLCVTLNCTDDVRNVNTNATNTNSSWGEPMKGEIKNCSFNITTSIRNKVQKQYALFYKLD
 VVPIDNDSNNNTNYRLISCNTSVITQACPKVSFEPPIPIHYCAPAGFAILKCNDKKFNGTGPCTNVSTVQCTHGIRPVV
 STQLLNLSLAEEVEVIRSENFTNNAKTIMVQLNVSVEINCTRPNNNTRKSIHIGPGRFYTAGDIIGDIRQAHCNI

SRANWNNTLRQIVEKLKGQFGNNKTIVFNHSSGGDPEIVMHSFNCGGEFFYCNSTKLFNSTWTWNNSTWNNTKRSND
 TEEHITLPCRIKQIINMWQEVGKAMYAPPPIRGQIRCSSNITGLLLTRDGGNDTSGTEIFRPGGGDMRDNRSELYKY
 KVKIEPLGVAPTAKRRVVQSEKSAVGIGAVFLGFLGAAGSTMGAASMTLTVQARLLLSGIVQQNNLLRAIEAQQ
 HLLQLTVWGIKQLQARVLAVERYLKDQQLLGIWGCSGKLICTTTVPWNASWSNKSOLDKIWNMTWMEWEREINNYTS
 LIYTLLIESONQOEK

SEQ ID NO: 9 (Mos2.Env, mosaic HIV envelope protein sequence; amino acids at positions (i)-(vii) for mutations according to the invention indicated by grey shading)

MGNLWVTVYYGVVPWKEAKTTLFCASDAKAYEKEVHNWATHACVPTDPNPQEMVLENTENFNMWKNMVDQMHE
 IIRLWDQSLKPCVKLTPLCVTLECRNVRNVSSNGTYNIIHNETYKEMKNCSFNATTVVEDRKQKVHALFYRLDIVPL
 DENNSSEKSSSENSSEYYRLINCNTSAITQACPQVSDPIPIHYCAPAGYAILKCNKTFNGTGPCNNVSTVQCTHGI
 KPVVSTQLLLNGSLAEIIIIRSENLTNNAKTIIVHLNETVNITCTRPNNNTRKSIRIGPGQTFYATGDIIGDIRQA
 HCNLSRDGWNKTLLQGVKKLAEHFPNKTINFTSSSGGDLEITTHSFNCRGFEFFYCNSTGLFNGTYMPNGTNSNSSN
 ITLPCRIKQIINMWQEVGRAMYAPPPIAGNITCRSNITGLLLTRDGGSNNGVPNDTETFRPGGGDMRNNRSELYKYK
 VVEVKPLGVAPTEAKRRVVEREKRAVGIGAVFLGILGAAGSTMGAASITLTVQARQLLSGIVQQSNLLRAIEAQQH
 MLQLTVWGIKQLQTRVLAIERYLQDQQLLGLWGCSGKLICTTAVPWNTSWSNKSQTDIWDNMTWMQWDKEIGNYTGE
 TYRLLIESONQOEK

SEQ ID NO: 10 (furin cleavage site mutant sequence)

RRRRRR

SEQ ID NO: 11 (example of a signal sequence (e.g. used for ConC_SOSIP, and some wild-type derived variants))

MRVRGILRNWQQWWIWIWGLGFWMMLMICNVVG (note: the last VG could be the beginning of the mature protein or the end of the signal sequence)

SEQ ID NO: 12 (example of 8 amino acid sequence that can replace HR1 loop)

NPDWLPDM

SEQ ID NO: 13 (example of 8 amino acid sequence that can replace HR1 loop)

GSGSGSGS

SEQ ID NO: 14 (example of 8 amino acid sequence that can replace HR1 loop)

DDVHPDWD

SEQ ID NO: 15 (example of 8 amino acid sequence that can replace HR1 loop)

RDTFALMM

SEQ ID NO: 16 (example of 8 amino acid sequence that can replace HR1 loop)

DEEKVMDF

SEQ ID NO: 17 (example of 8 amino acid sequence that can replace HR1 loop)

DEDPHWD

SEQ ID NO: 18 (example of a signal sequence (e.g. used for ConB_SOSIP))

MRVKGIRKNYQHLWRWGTMLLGMLMICA

SEQ ID NO: 19 (tag used for HIV gp140 constructs in AlphaLISA assay)

AAALPETGGGSDYKDDDDKPGGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGSHHHHHH

SEQ ID NO: 20 (stabilized ConC_SOSIP, 'ConC_SOSIP_7mut' (HIV160544))

NLWVTVYYGVPVWKEAKTTLFCASDAKAYEKEVHNWATHACVPTDPNPQEMVLENTENFNMWKNMVDQMHEDI I
 SLWDQSLKPCVKLTPLCVTLNCTNVNVTNTNNNNMKEEMKNC SFNTTTEIRDKKQKEYALFYRLDIVPLNENSSEYR
 LINCNTSTITQiCPKVSFDPIPIHYCAPAGYAILKCNKTFNGTGPCNNVSTVQCTHGIKPVVSTQLLLNGSLAEED
 I IIRSENLTDNAKTIIVHLNESVEINCTRPNNNTRKSIIRIGPGQTFYATGDIIGDIRQAHNCISEAKWNKTLQRVKK
 KLKEHFPNKTIKFAPSSGGDLEITTHSFNCRGEFFYCNTSKLFNSTYNNNTTSNSTITLPCRQIINMWQEVGRAMY
 APPIAGNITCKSNITGLLLTRDGGNNNNNTETFRPGGGMDRDNWRSELYKYKVVEIKPLGIAPTCKRRVVERRRRR
 RAVGIGAVFLGFLGAAGSTMGAASnTLTVQARQLLSGIVQQSNLLRAPEAQQHMLQLTVWGfKQLQARVLAIERYL
 evQQLLGIWGC SGKLI CCTAVPWNSSWSNKSQEDIWDMTWMQWDREISNYTDTIYRLLEESQfQQEiNEKDLLALD

SEQ ID NO: 21 (BG505_SOSIP Env protein (HIV150673))

AENLWVTVYYGVPVWKAETTLFCASDAKAYETEKHNWATHACVPTDPNPQEIHLNVTTEEFNMWKNNMVEQMHTD
 I IISLWDQSLKPCVKLTPLCVTLQCTNVNNTITDDMRGELKNC SFNMTTEL RDKKQKVYSLFYRLDVVQINENQGNRS
 NNSNKEYRLINCNTSAITQACPKVSFEPIPIHYCAPAGFAILKCKDKKFNGTGPCPSVSTVQCTHGIKPVVSTQLLL
 NGSLAEEDVMIRSENITNNAKNILVQFNTVPQINCTRPNNNTRKSIIRIGPGQAFYATGDIIGDIRQAHNCVSKATWN
 ETLGKVVQQLRKHFNNITIRFANSSGGDLEVTTHSFNCGGEFFYCNTSGLFNSTWISNTSVQGSNSTGSNDSTITLP
 CRIKQIINMWQRIGQAMYAPPIQGVIRCVSNITGLILTRDGGSTNSTTETFRPGGGMDRDNWRSELYKYKVVKIEPL
 GVAPTRCKRRVVGRRRRRAVGIGAVFLGFLGAAGSTMGAASMTLTVQARNLLSGIVQQSNLLRAPEAQQHLLKLT
 VWGIKQLQARVLAVERYLRDQQLLGIWGC SGKLI CCTNVPWNSSWSNRNLSEIWDNMTWLQWDKEISNYTQI IYGLL
 EESQNQQEKNEQDILLALD

SEQ ID NO: 22 (stabilized BG505_SOSIP Env protein (HIV170863))

AENLWVTVYYGVPVWKAETTLFCASDAKAYETEKHNWATHACVPTDPNPQEIHLNVTTEEFNMWKNNMVEQMHTD
 I IISLWDQSLKPCVKLTPLCVTLQCTNVNNTITDDMRGELKNC SFNMTTEL RDKKQKVYSLFYRLDVVQINENQGNRS
 NNSNKEYRLINCNTSAITQACPKVSFEPIPIHYCAPAGFAILKCKDKKFNGTGPCPSVSTVQCTHGIKPVVSTQLLL
 NGSLAEEDVMIRSENITNNAKNILVQFNTVPQINCTRPNNNTRKSIIRIGPGQAFYATGDIIGDIRQAHNCVSKATWN
 ETLGKVVQQLRKHFNNITIRFANSSGGDLEVTTHSFNCGGEFFYCNTSGLFNSTWISNTSVQGSNSTGSNDSTITLP
 CRIKQIINMWQRIGQAMYAPPIQGVIRCVSNITGLILTRDGGSTNSTTETFRPGGGMDRDNWRSELYKYKVVKIEPL
 GVAPTRCKRRVVGRRRRRAVGIGAVFLGFLGAAGSTMGAASnTLTVQARNLLSGIVQQSNLpRAPEAQQHLLKLT
 VWGIKQLQARVLAVERYLRvQQLLGIWGC SGKLI CCTNVPWNSSWSNRNLSEIWDNMTWLQWDKEISNYTQI IYGLL
 EESQfQQEiNEQDILLALD

SEQ ID NO: 23 (wt C97ZA_SOSIP Env protein with L535M and Q567K (HIV150673))

NMWVTVYYGVPVWTDAKTTLFCASDTKAYDREVHNWATHACVPTDPNPQEIIVLENTENFNMWKNMVDQMHEDI I
 SLWDQSLKPCVKLTPLCVTLHCTNATFKNNVTNDMNKEIRNCSFNNTTTEIRDKKQQGYALFYRPDIVLLKENRNNNS
 NSEYILINCNASTITQACPKVNFDPPIPIHYCAPAGYAILKCNKTFSGKGPCNNVSTVQCTHGIKPVVSTQLLLNGS
 LAEKEIIIRSENLTDNVKTIIIVHLNKSVEIVCTRPNNNTRKSMRIGPGQTFYATGDIIGDIRQAYCNISGSKWNETL
 KRVKEKLQENYNNNKTIKFAPSSGGDLEITTHSFNCRGEFFYCNTTRLFNNNATEDETITLPCRQIINMWQGVGR
 AMYAPPIAGNITCKSNITGLLLVRDGGEDNKTEEIFRPGGGNMKDNWRSELYKYKVIELKPLGIAPTGcKRRVVERr
 rrrRAVGIGAVFLGFLGAAGSTMGAASmTLTVQARQLLSIVQQSNLLRApEAQQHMLkLTVWGfKQLQTRVLAIE
 RYLDKQQLLGIWGC SGKLI CCTNVPWNSSWSNKSQTDIWNMTWMEWDREISNYTDTIYRLLEDSTQfQQEKNEKDLL
 ALD

SEQ ID NO: 24 (repaired and stabilized C97ZA_SOSIP Env protein (HIV170690))

NLWVTVYYGVPVWkDAKTTLFCASDaKAYDREVHNWATHACVPTDPNPQEIIVLENTENFNMWKNMVDQMHEDI I
 SLWDQSLKPCVKLTPLCVTLHCTNATFKNNVTNDMNKEIRNCSFNNTTTEIRDKKQkvYALFYRLDIVqLKENRNNNS
 NSEYrLINCNTSTITQiCPKvtFDPIPIHYCAPAGYAILKCNKTFnGtGPCNNVSTVQCTHGIKPVVSTQLLLNGS
 LAEKEIIIRSENLTDNVKTIIIVHLNKSVEInCTRPNNNTRKSiRIGPGQTFYATGDIIGDIRQAYCNISGSKWNETL
 KRVKEKLrEhfNNNKTIKFAPSSGGDLEITTHSFNCRGEFFYCNTTRLFNNNATENeTITLPCRQIINMWQeVGR
 AMYAPPIAGNITCKSNITGLLLtRDGGEDNKTEEIFRPGGGNMKDNWRSELYKYKVvEiKPLGIAPTkcKRRnVtRr
 rrrRAVGIGAVFLGFLGAAGSTMGAASnTLTVQARQLLSgIVQQSNLpRApEAQQHMLkLTVWGfKQLQaRVLAIE

RYLevQQLLGIWGC SGKLICcTNVPWNSSWSNKSQTDIWNMTWMEWDREISNYTDTIYRLLEDSQfQQEiNEKDLL
AnD

SEQ ID NO: 25 (variant of repaired and stabilized Du422 construct (HIV161818))

NLWVTVYYGVPVWKEAKTTLFCASDAKAYDKEVHNVWATHACVPTDPNPQEIVLENTENFNMWKNMVDQMHEDI
SLWDQSLKPCVKLTPLCVTLNCKNVNISANANATATLNSMNGEIKNCSFNNTTTEL RDKKQKVYALFYKPDVVP LNG
GEHNETGEYILINCNSSTcTQACPKVSFDPIPIHYCAPAGYAILKCNKTFNGTGPCNNVSTVQCTHGIKPVVSTQL
LLNGSLAEEEEIIIRSENLTNNIKTIIIVHLNKSVEINCTRPNNNTRKSVRIGPGQTFYATGEIIGDIREAHCNISRET
WNSTLIQVKEKLEHYNKTIFEPSSGGDLEVTTHSFNCRGEFFYCNTTKLFNETKLFNESEYVDNKTIIILPCRIKQ
IINMWQEVGRcMYAPPIEGNITCKSNITGLLLTRDGGENSTEEVFRPGGGMKDNWRSELYKYKVVEIKPLGVAPTK
CKRKnVtRRRRRRRAVGLGAVLLGFLGAAGSTMGAASnTLTVQARQLLSGIVQQQSNLpRAPEAQQHLLQLTVWGIKQ
LQTRVLAIERYLkVQQLLGLWGC SGKLICCTAVPWNSSWSNKS LGDIWDNMTWMQWDREISNYTNTIYRLLEDSQfQ
QEKNEKDLLAnD

SEQ ID NO: 26 (repaired and stabilized Du422_SOSIP (HIV170859))

NLWVTVYYGVPVWKEAKTTLFCASDAKAYDKEVHNVWATHACVPTDPNPQEIVLENTENFNMWKNMVDQMHEDI
SLWDQSLKPCVKLTPLCVTLNCKNVNISANANATATLNSMNGEIKNCSFNNTTTEL RDKKQKVYALFYKPDVVP LNG
GEHNETGEYILINCNSSTITQACPKVSFDPIPIHYCAPAGYAILKCNKTFNGTGPCNNVSTVQCTHGIKPVVSTQL
LLNGSLAEEEEIIIRSENLTNNIKTIIIVHLNKSVEINCTRPNNNTRKSVRIGPGQTFYATGEIIGDIREAHCNISRET
WNSTLIQVKEKLEHYNKTIFEPSSGGDLEVTTHSFNCRGEFFYCNTTKLFNETKLFNESEYVDNKTIIILPCRIKQ
IINMWQEVGRAMYAPPIEGNITCKSNITGLLLTRDGGENSTEEVFRPGGGMKDNWRSELYKYKVVEIKPLGVAPTK
CKRKVVGRRRRRRAVGLGAVLLGFLGAAGSTMGAASnTLTVQARQLLSGIVQQQSNLpRAPEAQQHLLQLTVWGIKQ
LQTRVLAIERYLkVQQLLGLWGC SGKLICCTAVPWNSSWSNKS LGDIWDNMTWMQWDREISNYTNTIYRLLEDSQfQ
QEiNEKDLLALD

SEQ ID NO: 27 (repaired and stabilized DS_sC4_SOSIP (HIV170686))

MGNLWVTVYYGVPVWKDAKTTLFCASDAKAYEKEVHNVWATHACVPTDPNPQEIVLGNVTENFNMWKNMVDQMHE
IISLWDqSLkPCVKLTPLCVTLNCRNVRNEMKNCSFNATTVVrDRKQKVHALFYRLDIVPLDENSSYRLINCNTS
AcTQiCPKVSFDPIPIHYCAPAGYAILKCNKTFNGTGPCNNVSTVQCTHGIKPVVSTQLLLNGSLAEEEEIIIRSEN
LTNNAKTIIVHLNETVNICTRPNNNTRKSVRIGPGQTFYATGDIIGDIRQAHCNLSRDGWNKTLQGVKKKLAEHFP
NKTIFKAPHSGGDLEITTHSFNCRGEFFYCNTSNLFNESNIERNDSIITLPCRIKQIINMWQEVGRcmYAPPIAGNI
TCRSNITGLLLTRDGGSNNDTETFRPGGDMRNWRSELYKYKVVEVKPLGVAPTECKRRVVERRRRRAVGIGAV
FLGILGAAGSTMGAASnTLTVQARQLLSGIVQQQSNLpRAPEAQQHMLQLTVWGIKQLQTRVLAIERYLkVQQLLGL
WGCSGKLICCTAVPWNTSWSNKSQTDIWDNMTWMQWDKEIGNYTGEIYRLLEESQfQQEi

SEQ ID NO: 28 (Stabilized ConC_SOSIP.v3 (HIV170654))

NLWVTVYYGVPVWKEAKTTLFCASDAKAYEKEVHNVWATHACVPTDPNPQEMVLENTENFNMWKNMVDQMHEDI
SLWDQSLKPCVKLTPLCVTLNCTNVNVTMKNCSFNNTTEIRDKKQKEYALFYRLDIVPLNENSSEYRLINCNTSTI
TQICPKVSFDPIPIHYCAPAGYAILKCNKTFNGTGPCNNVSTVQCTHGIKPVVSTQLLLNGSLAEEEEIIIRSENLT
NNVKTIIIVHLNESVEIVCTRPNNNTRKSVRIGPGQTFYATGDIIGDIRQAHCNISEAKWNKTLQRVKKKLEHFPNK
TIKFQPSGGDLEITTHSFNCRGEFFYCNTSKLFNSTYNNTTSNSTITLPCRIKQIINMWQEVGRAMYAPPIAGNIT
CKSNITGLLLTRDGGNNNNNTETFRPGGDMRDNRSELYKYKVVEIKPLGIAPTKCKRRVVERRRRRAVGIGAVF
LGFLGAAGSTMGAASnTLTVQARQLLSGIVQQQSNLLRAPEAQQHMLQLTVWGFQQLQARVLAIERYLEVQQLLGIW
GCSGKLICCTAVPWNSSWSNKSQEDIWDNMTWMQWDREISNYTDTIYRLLEESQfQQEINEKDLLALD

SEQ ID NO: 29 (Stabilized BG505_SOSIP.v2 (HIV171814))

AENLWVTVYYGVPVWKDAETTTLFCASDAKAYETEKHNWATHACVPTDPNPQEIHLNVTTEEFNMWKNNMVEQMHTD
IISLWDQSLKPCVKLTPLCVTLQCTNVNNTIIDD MRGELKNCSFNMTTEL RDKKQKVYSLFYRLDVVQINENQGNRS
NNSNKEYRLINCNTSAcTQACPKVSFEPIPIHYCAPAGFAILKCKDKKFNGTGPCPSVSTVQCTHGIKPVVSTQLLL
NGSLAEEVVMIRSENITNNAKNILVQFNTPVQINCTRPNNNTRKSVRIGPGQAFYATGDIIGDIRQAHCNVSKATWN
ETLGKVVQQLRKHFNNITIRFANSSGGDLEVTTHSFNCGGEFFYCNTSGLFNSTWISNTSVQGSNSTGSNDSITLP
CRIKQIINMWQRIGQcMYAPPIQGVIRCVSNITGLIILTRDGGSTNSTTETFRPGGDMRDNRSELYKYKVVEIKPL
GVAPTRCKRRVVGRRRRRRAVGIGAVFLGFLGAAGSTMGAASnTLTVQARNLLSGIVQQQSNLpRAPEAQQHLLKLT

VWGIKQLQARVLAVERYLevQQLLGIWGCSGKLICCTNVPWNSSWSNRNLSEIWDNMTWLQWDKEISNYTQIIYGLLEESQfQQEiNEvDLLALD

SEQ ID NO: 30 (Repaired and stabilized C97ZA_SOSIP.v2 (HIV171810))

NLWVTVYYGVPVWkDAKTTLFCASDaKAYDREVHNWATHACVPTDPNPQEIVLENTENFNMWKNMVDQMHEDIISLWDQSLKPCVKLTPLCVTLHCTNATFKNNVTNDMNKEIRNCSFNNTTTEIRDKKQkvYALFYRLDIVqLKENRNNSN NSEYrLINCNTStcTQiCPKVtFDPIPIHYCAPAGYAILKCNKTFnGtGPCNNVSTVQCTHGIKPVVSTQLLNGSLAEKEIIIRSENLTDNVKTIIIVHLNKSVEInCTRPNNNTRKSiRIGPGQTFYATGDIIGDIRQAYCNISGSKWNETL KRVKEKLrEhfNNNKTIKFAPSSGGDLEITTHSFNCRGEFFYCNTTRLFNNNATENETITLPCRIKQIINMWQeVGR cMYAPPIAGNITCKSNITGLLLtRDGGEDNKTEEIFRPGGGNMKDNWRSELYKYKVvEiKPLGIAPTkcKRRnVtRr rrrRAVGIGAVFLGFLGAAGSTMGAASnTLTVQARQLLSgIVQQQSNLpRApEAQQHMLkLTVWGIKQLQaRVLAIE RYLevQQLLGIWGCSGKLICcTNVPWNSSWSNKSQTDIWNMTWMEWDREISNYTDTIYRLLEDSQfQQEiNEvDLL AnD

SEQ ID NO: 31 (Repaired and stabilized Du422_SOSIP.v1 (HIV171812))

NLWVTVYYGVPVWKEAKTTLFCASDAKAYDKEVHNWATHACVPTDPNPQEIVLENTENFNMWKNMVDQMHEDIISLWDQSLKPCVKLTPLCVTLNCKNVNISANANATATLNSMNGEIKNCSFNNTTTELDRKKQKVYALFYKPDVPLNG GEHNETGEYILINCNSStcTQACPKVSFDPIPIHYCAPAGYAILKCNKTFNGTGPCNNVSTVQCTHGIKPVVSTQL LLNGSLAEKEIIIRSENLTNNIKTIIVHLNKSVEINCTRPNNNTRKSVRIGPGQTFYATGEIIGDIREAHCNISRET WNSTLIQVKEKLREHYNKTIKFEPSSGGDLEVTTHSFNCRGEFFYCNTTKLFNETKLFNESEYVDNKTIILPCRIKQ IINMWQEVGRcMYAPPIEGNITCKSNITGLLLTRDGGENSTEEVFRPGGGNMKDNWRSELYKYKVVEIKPLGVAPTCKRKVVGRRRRRRAVGLGAVLLGFLGAAGSTMGAASnTLTVQARQLLSGIVQQQSNLpRAPEAQHLLQLTVWGIKQLQTRVLAIERYLEvQQLLGLWGCSGKLICCTAVPWNSSWSNKSGLDIWDNMTWMQWDREISNYTNTIYRLLEDSQfQQEiNEvDLLALD

SEQ ID NO: 32 (Stabilized and repaired sC4_SOSIP.v4)

MGNLWVTVYYGVPVWkDAKTTLFCASDAKAYEKEVHNWATHACVPTDPNPQEIVLGNVTENFNMWKNMVDQMHEDIISLWDqSLkPCVKLTPLCVTLNCRNVRNEMKNCSFNATTVVrDRKQKVHALFYRLDIVPLDENSSYRLINCNTS ActQiCPKVSFDPIPIHYCAPAGYAILKCNKTFNGTGPCNNVSTVQCTHGIKPVVSTQLLNGSLAEKEIIIRSEN LTNNAKTIIVHLNETVNIVCTRPNNNTRKSiRIGPGQTFYATGDIIGDIRQAHCNLSRDGWNKTLQGVKKLAEHFP NKTIKFAPHSGGDLEITTHSFNCRGEFFYCNTSNLFENSIERNDSIITLPCRIKQIINMWQEVGRcmYAPPIAGNITCRSNITGLLLTRDGGSNMNDTETFRPGGDMRNWRSELYKYKVVEVKPLGVAPTECKRRnVtRRRRRRRAVGIGAV FLGILGAAGSTMGAASnTLTVQARQLLSGIVQQQSNLpRAPEAQHMLQLTVWGIKQLQTRVLAIERYLEdQQLLGL WGCSGKLICCTAVPWNTSWSNKSQTDIWDNMTWMQWDKEIGNYTGEIYRLLEESQfQQEi

SEQ ID NO: 33 (example of a signal sequence (e.g. used for DS_sC4_SOSIP variants))

MRVRGMLRNWQQWWIWSSLGFWMLMIYSV

SEQ ID NO: 34 (example of a signal sequence (e.g. used for BG505_SOSIP variants))

MRVMGIQRNCQHLEFRWGTMLGMIITCSA

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CLAIMS

What is claimed is:

1. A recombinant human immunodeficiency virus (HIV) envelope (Env) protein, comprising two or more of the following amino acid residues:

- (i) Phe, Leu, Met, or Trp, preferably Phe, at position 651;
- (ii) Phe, Ile, Met, or Trp, preferably Ile, at position 655;
- (iii) Asn or Gln, preferably Asn, at position 535;
- (iv) Val, Ile or Ala, preferably Val, at position 589;
- (v) Phe or Trp, preferably Phe, at position 573;
- (vi) Ile at position 204; and/or
- (vii) Phe, Met, or Ile, preferably Phe, at position 647,

wherein the numbering of the positions is according to the numbering in gp160 of HIV-1 isolate HXB2.

2. A recombinant HIV Env protein, comprising one or more of the following amino acid residues:

- (i) Phe, Leu, Met, or Trp, preferably Phe, at position 651;
- (ii) Phe, Ile, Met, or Trp, preferably Ile, at position 655;
- (iii) Asn or Gln, preferably Asn, at position 535;
- (iv) Val, Ile or Ala, preferably Val, at position 589;
- (v) Phe or Trp, preferably Phe, at position 573;
- (vi) Ile at position 204; and/or
- (vii) Phe, Met, or Ile, preferably Phe, at position 647,

wherein the HIV Env protein is selected from the group consisting of:

- (1) an HIV Env consensus sequence, for instance from clade C for instance comprising the amino acid sequence of SEQ ID NO: 2 or 3 or for instance from clade B for instance comprising the amino acid sequence of SEQ ID NO: 4 or 5;
- (2) a synthetic HIV Env protein, for instance comprising the amino acid sequence of (a): SEQ ID NO: 6; or (b): SEQ ID NO: 6 with a mutation of Glu to Arg at position 166; or (c): (a) or (b) with a mutation of the amino acids at positions 501 and 605 into Cys residues and a mutation of the amino acid at position 559 into a Pro residue; or (d): (a), (b) or (c) having a further furin cleavage site mutation,

e.g. replacement of the amino acids at positions 508-511 by RRRRRR (SEQ ID NO: 10); or (e) SEQ ID NO: 7; or (f) SEQ ID NO: 8 or SEQ ID NO: 9; and

- (3) a parent HIV Env protein which is preferably a wild-type HIV Env protein, preferably of clade C, comprising at least one repair mutation at an amino acid residue that is present at the corresponding position at a frequency of less than 7.5%, preferably less than 2%, of HIV Env sequences in a collection of at least 1000, preferably at least 10000, wild-type HIV Env sequences, wherein the repair mutation is a substitution by an amino acid residue that is present at the corresponding position at a frequency of at least 10% of HIV Env sequences in said collection and preferably the repair mutation is a substitution by the amino acid residue that is present at the corresponding position most frequently in said collection;
- and

wherein the numbering of the positions is according to the numbering in gp160 of HIV-1 isolate HXB2.

3. A recombinant HIV Env protein, comprising one or more of the following amino acid residues:

- (i) Phe, Leu, Met, or Trp, preferably Phe, at position 651;
- (ii) Phe, Ile, Met, or Trp, preferably Ile, at position 655;
- (iii) Asn or Gln, preferably Asn, at position 535;
- (iv) Val, Ile or Ala, preferably Val, at position 589;
- (v) Phe or Trp, preferably Phe, at position 573;
- (vi) Ile at position 204; and/or
- (vii) Phe, Met, or Ile, preferably Phe, at position 647,

wherein the HIV Env protein is a HIV Env protein comprising at least one of the following:

- (a) Cys at positions 501 and 605;
- (b) Pro at position 559;
- (c) Cys at positions 501 and 605 and Pro at position 559; and

the numbering of the positions is according to the numbering in gp160 of HIV-1 isolate HXB2.

4. The recombinant HIV Env protein of claim 2 or 3, comprising two or more of the amino acid residues indicated in (i) to (vii).
5. The recombinant HIV Env protein of any of claims 1, 2, and 4, comprising Cys at positions 501 and 605 or Pro at position 559, preferably Cys at positions 501 and 605 and Pro at position 559.
6. The recombinant HIV Env protein of any of claims 1 to 5, comprising three or more of the amino acid residues indicated in (i) to (vii).
7. The recombinant HIV Env protein of any of claims 1 to 5, comprising four or more of the amino acid residues indicated in (i) to (vii).
8. The recombinant HIV Env protein according to any one of claims 1 to 7, comprising Phe at position 651, Ile at position 655, Asn at position 535, and Val at position 589.
9. The recombinant HIV Env protein of any of claims 1 to 8, comprising five or more of the amino acid residues indicated in (i) to (vii).
10. The recombinant HIV Env protein of any of claims 1 to 9, further comprising one or more of the following:
 - (viii) Gln, Glu, Ile, Met, Val, Trp, or Phe, preferably Gln or Glu, at position 588;
 - (ix) Lys at position 64 or Arg at position 66 or Lys at position 64 and Arg at position 66;
 - (x) Trp at position 316;
 - (xi) Cys at both positions 201 and 433;
 - (xii) Pro at position 556 or 558 or at both positions 556 and 558;
 - (xiii) replacement of the loop at amino acid positions 548-568 (HR1-loop) by a loop having 7-10 amino acids, preferably a loop of 8 amino acids, for example having a sequence chosen from any one of (SEQ ID NOs: 12-17);
 - (xiv) Gly at position 568, or Gly at position 569, or Gly at position 636, or Gly at both positions 568 and 636, or Gly at both positions 569 and 636; and/or
 - (xv) Tyr at position 302, or Arg at position 519, or Arg at position 520, or Tyr at position 302 and Arg at position 519, or Tyr at position 302 and Arg at position 520, or Tyr at position 302 and Arg at both positions 519 and 520.

11. The recombinant HIV Env protein of any of claims 1 to 10, further comprising a mutation in a furin cleavage sequence of the HIV Env protein, preferably a replacement at positions 508-511 by RRRRRR (SEQ ID NO: 10).
12. The recombinant HIV Env protein of any of claims 1 to 11, comprising an amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 3, 5, 20, 22, 24, 26, 27, 28, 29, 30, 31, or 32.
13. The recombinant HIV Env protein of any of claims 1 to 12, further comprising: (xvi) an amino acid residue chosen from Val, Ile, Phe, Met, Ala, or Leu, preferably Val or Ile, most preferably Val, at position 658.
14. The recombinant HIV Env protein of any of claims 1 to 13, being a gp140 or gp160 protein.
15. The recombinant HIV Env protein of any of claims 1 to 14, which is from clade C or clade A, preferably from clade C.
16. A trimeric complex comprising a noncovalent oligomer of three of the recombinant HIV Env proteins of any of claims 1 to 15.
17. A particle, preferably a liposome or nanoparticle, displaying on its surface a recombinant HIV Env protein of any of claims 1 to 15 or a trimeric complex of claim 16.
18. An isolated nucleic acid molecule encoding a recombinant HIV Env protein of any of claims 1 to 15.
19. A vector comprising the isolated nucleic acid molecule of claim 18 operably linked to a promoter.
20. The vector of claim 19, which is an adenovirus vector.
21. A host cell comprising the isolated nucleic acid molecule of claim 18 or the vector of claim 19 or 20.
22. A method of producing a recombinant HIV Env protein, comprising growing the host cell of claim 21 under conditions suitable for production of the recombinant HIV Env protein.
23. A composition comprising the recombinant HIV Env protein of any of claims 1 to 15, the trimeric complex of claim 16, the particle of claim 17, the isolated nucleic acid molecule of claim 18, or the vector of claim 19 or 20, and a pharmaceutically acceptable carrier.
24. A method of improving the trimer formation of an HIV Env protein, the method comprising substituting one or more amino acid residues in a parent HIV Env protein, wherein the one or more substitutions result in one or more of the following amino acids:

- (i) Phe, Leu, Met, or Trp, preferably Phe, at position 651;
- (ii) Phe, Ile, Met, or Trp, preferably Ile, at position 655;
- (iii) Asn or Gln, preferably Asn, at position 535;
- (iv) Val, Ile or Ala, preferably Val, at position 589;
- (v) Phe or Trp, preferably Phe, at position 573;
- (vi) Ile at position 204; and/or
- (vii) Phe, Met, or Ile, preferably Phe, at position 647,

wherein the numbering of the positions is according to the numbering in gp160 of HIV-1 isolate HXB2.