SOSIP Changes Affect Human Immunodeficiency Virus Type 1 Envelope Glycoprotein Conformation and CD4 Engagement

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ABSTRACT The entry of human immunodeficiency virus into host cells is mediated by the envelope glycoprotein (Env) trimeric spike, which consists of three exterior gp120 subunits and three transmembrane gp41 subunits. The trimeric Env undergoes extensive conformational rearrangement upon interaction with the CD4 receptor, transitioning from the unliganded, “closed” State 1 to more-open downstream State 2 and State 3 conformations. Changes in “restraining” amino acid residues, such as leucine 193 and isoleucine 423, destabilize State 1 Env, which then assumes entry-competent, downstream conformations. The introduction of an artificial disulfide bond linking the gp120 and gp41 subunits (SOS) in combination with the I559P (IP) change has allowed structural characterization of soluble gp140 (sgp140) trimers. The conformation of these SOSIP-stabilized sgp140 trimers has been suggested to represent the closed native State 1 conformation. Here we compare the impact on the membrane Env conformation of the SOSIP changes with that of the well-characterized changes (L193R and I423A) that shift Env to downstream States 2 and 3. The results presented here suggest that the SOSIP changes stabilize Env in a conformation that differs from State 1 but also from the downstream Env conformations stabilized by L193R or I423A.

IMPORTANCE The human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein (Env) trimer is triggered by receptor binding to mediate the entry of the virus into cells. Most structural studies of Env trimers have utilized truncated soluble gp140 Envs stabilized with the I559P and SOS changes. Here we present evidence indicating that these stabilizing changes have a profound impact on the conformation of Env, moving Env away from the native pretriggered Env conformation. Our studies underscore the need to acquire structural information on the pretriggered Env conformation, which is recognized by most broadly reactive neutralizing antibodies.

KEYWORDS Env, Env conformation, HIV-1, SOSIP, State 1, States 2/3

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The entry of human immunodeficiency virus type 1 (HIV-1) into the host cell is mediated by the viral envelope glycoproteins (Env), which are derived by proteolytic cleavage of a trimeric gp160 Env precursor (1–3). The mature Env complex is composed of three gp120 surface subunits and three gp41 transmembrane subunits. Receptor binding drives the metastable Env trimer from its unliganded high-energy conformation (State 1) to an “open,” CD4-bound, lower-energy conformation (State 3). CD4 engagement initially results in an intermediate, “partially open” Env conformation (State 2); the binding of additional CD4 molecules induces State 3, the prehairpin intermediate conformation (4, 5). In the State 3 conformation, gp120 is competent for binding the coreceptor and the gp41 heptad repeat (HR1) coiled coil is formed and exposed. Binding of the State 3 Env to the coreceptor, either CCR5 or CXCR4, is required for fusion of the viral and target cell membranes (1, 6–14), as recently reviewed (15).

In addition to its role in viral entry, Env conformational flexibility also contributes to the ability of HIV-1 to evade the host antibody response. Envs from primary HIV-1 are restrained in the high-energy State 1 conformation, which renders them more resistant to soluble CD4 (sCD4) and to neutralizing antibodies. Multiple intramolecular and/or intermolecular interactions within the Env trimer are thought to contribute to the maintenance of the State 1 conformation. Recent mutagenesis studies identified several gp120 V1V2 and β 20– β 21 residues that stabilize Env in State 1. Alteration of these “restraining” residues frees Env to sample the increasingly lower energy States 2 and 3 (4, 16). Accordingly, these variants are more sensitive to neutralization by State 2- and State 3-specific ligands, such as sCD4, small CD4-mimetic compounds (CD4mc), and CD4-induced (CD4i) antibodies, including the coreceptor binding site (CoRBS) antibody 17b (4, 16).

While Env metastability is essential for membrane fusion and viral entry, it creates major challenges for the expression and structural analysis of trimeric Env. The introduction of an artificial disulfide bond (SOS) linking the gp120 and gp41 subunits (17–20), in combination with the helix-breaking isoleucine-to-proline change (I559P) in the gp41 ectodomain (IP) (20, 21), permitted structural characterization of cleaved soluble SOSIP gp140s (22–25). A high-resolution structure of the trimeric HIV-1 Env ectodomain was also obtained with a cytoplasmic-tail-deleted, membrane-bound Env in complex with antibody PGT151 (26). These Env trimer structures are similar and exhibit a central gp41 HR1 coiled coil, from which emanates a three-bladed propeller composed of gp120 subunits; these structures have been proposed to represent the “closed,” pretriggered State 1 conformation (22–26). However, other studies indicated that the I559P change stabilizes the trimeric membrane-anchored Env in a conformation that differs from State 1 (27, 28). To understand better the impact of the SOSIP changes on Env conformation, we compare Env mutants with these changes and with two well-characterized State 2-stabilizing changes (L193R and I423A) in gp120 (4, 16). The Env mutants were evaluated for their abilities to interact with the CD4 receptor as well as with ligands that exhibit preferences for particular Env conformational states.

RESULTS

Effects of L193R and I423A mutations on the conformation of HIV-1_{BG505} Env.

We recently reported a series of State 1-restraining residues located in the gp120 V1V2 and β 20– β 21 regions (4, 16); when these residues are altered, Env is free to sample downstream conformations spontaneously. Based on their marked State 2/3 phenotypes (4, 16), the L193R and I423A changes were selected and introduced into the clade A HIV-1_{BG505} Env, which has been widely used for structural analysis of SOSIP-stabilized soluble gp140 (sgp140) trimers (22–25, 29, 30). To confirm that these changes shift HIV-1_{BG505} Env into States 2/3, recombinant luciferase-expressing viral particles bearing wild-type Env or its L193R or I423A variant were produced and evaluated for susceptibility to Env ligands that prefer State 1 (VRC03, PG9, and PGT151) or States 2 and 3 (sCD4 and 17b) (4, 16, 31). The wild-type HIV-1_{BG505} Env was inhibited by antibodies VRC03, PG9, and PGT151 but was highly resistant to ligands sCD4 and 17b (Fig. 1). This phenotype is consistent with the wild-type BG505 Env primarily occupying State 1.

FIG 1 Sensitivities of viruses with Env variants to neutralization by some State 1- and State 2/3-preferring ligands. Normalized amounts of recombinant luciferase-expressing HIV-1 pseudotyped with the wild-type (wt), L193R, or I423A HIV-1_{BG505} Env were incubated at 37°C with increasing concentrations of the State 1-preferring ligand VRC03 (A), PG9 (B), or PGT151 (C) or the State 2/3-preferring ligand sCD4 (D) or 17b (E). Data are representative of results obtained in at least three independent experiments, performed in quadruplicate.

Compared with wild-type HIV-1_{BG505}, both the L193R and I423A mutants were relatively resistant to VRC03, PG9, and PGT151 and relatively susceptible to sCD4 and 17b. Of note, the I423A mutation disrupts the 17b epitope (16) and therefore was not tested for neutralization by 17b. The sensitivity/resistance phenotypes of the L193R and I423A mutants are consistent with these Envs increasingly sampling State 2/3 conformations (Fig. 1). To confirm the State 2/3-stabilized phenotype of the L193R and I423A mutants, we expressed Envs containing these changes on cells and evaluated their conformations by a cell-based enzyme-linked immunosorbent assay (ELISA) using the following conformation-specific ligands: PG9, VRC03, b12, and PGT151 (State 1 preferring); sCD4, F105, 17b, 19b, GE2 JG8, F240, and 7b2 (State 2/3 preferring) (4, 16, 31). As shown in Fig. 2, State 1-preferring ligands PG9, VRC03, b12, and PGT151 recognized the L193R and I423A mutants less efficiently than the wild-type HIV-1_{BG505} Env. This was concomitant with a marked increase in the recognition of these mutants by State 2/3-preferring ligands. These observations suggest that the L193R and I423A changes shift the distribution of Env conformations toward State 2/3.

FIG 2 Ligand binding to membrane-anchored wild-type HIV-1_{BG505} Env and its L193R and I423A variants. The binding of the indicated HIV-1_{BG505} Env variants expressed on the cell surface was measured using a cell-based ELISA. The means and standard errors of the means (SEM) derived from at least five independent experiments performed in quadruplicate are reported. Statistical significance was evaluated using multiple *t* tests, correcting for multiple comparisons using the Holm-Sidak method (**, *P* < 0.01; ***, *P* < 0.001; ****, *P* < 0.0001; ns, not significant).

FIG 3 Impact of SOSIP mutations on ligand binding to membrane-anchored HIV-1_{BG505} Env variants. The binding of the indicated ligands to HIV-1_{BG505} Env variants expressed on the cell surface was measured using a cell-based ELISA. The means and SEM derived from at least five independent experiments performed in quadruplicate are reported. Statistical significance was evaluated using an unpaired Student *t* test or a Mann-Whitney test (*, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; ****, *P* < 0.0001; ns, not significant).

Effects of SOSIP mutations on HIV-1_{BG505} Env conformation. We reported previously that introduction of the I559P change results in major conformational changes in Envs expressed on the cell surface, relative to wild-type Envs (27). Indeed, we found that I559P Envs exhibited a relative decrease in recognition by certain State 1-preferring ligands (PG9, b12, and PGT151) (27). Here we evaluated the impact of the SOSIP mutations on HIV-1_{BG505} Env on the surface of cells and compared its effect to those of the State 2-stabilizing L193R and I423A changes. As reported previously (27), we found that introduction of the SOSIP changes significantly decreased recognition by State 1-preferring ligands (PG9, b12, and PGT151) but also decreased recognition by all State 2/3-preferring ligands tested (Fig. 3A). When the SOSIP mutant was compared with the

FIG 4 Exposure of the coreceptor binding site on HIV-1_{BG505} Env variants. 293T cells were transfected with an empty pcDNA3.1 plasmid or a plasmid expressing one of the indicated HIV-1_{BG505} Env variants. Two days posttransfection, Env expression was evaluated with antibody 2G12, which recognizes the gp120 outer domain independently of Env conformation. The spontaneous exposure of the 17b epitope, which overlaps the coreceptor binding site of gp120 (32), on the indicated Env variants is shown relative to 2G12 binding. Data represent the means and SEM from at least four independent experiments, presented as the mean fluorescence intensity (MFI) detected with MAb 17b divided by the MFI detected by antibody 2G12. Statistical significance was evaluated using an unpaired Student *t* test or a Mann-Whitney test (*, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; ****, *P* < 0.0001; ns, not significant).

two well-characterized L193R and I423A mutants (4, 16), we also observed major differences in ligand recognition. SOSIP Env was better recognized by antibodies PG9, VRC03, and PGT151 but interacted less efficiently with all State 2/3-preferring ligands tested (Fig. 3B). Taken together, these data are consistent with the SOSIP changes stabilizing Env in a conformation that differs from those of the wild-type, L193R, and I423A Envs.

Since the SOSIP and L193R or I423A changes resulted in opposite phenotypes with respect to recognition by certain ligands (sCD4, F105, 17b, 19b, GE2 JG8, F240, and 7b2), we evaluated whether a combination of either the L193R or I423A change with the SOSIP alterations was sufficient to restore a wild-type level of recognition by these ligands. The combination of SOSIP changes with the L193R or I423A change enhanced recognition by some State 2/3-preferring ligands (sCD4, 17b, 19b) to levels significantly higher than those observed for the SOSIP mutant and, in some cases, for the individual L193R or I423A mutant (Fig. 3C). As expected, SOSIP Envs were not recognized by anti-gp41 antibodies F240 and 7b2, in agreement with the conformational changes induced by I559P (27, 28). Lack of recognition of I423A Envs by 17b likely results from the partial disruption of the 17b epitope by this change (16, 32). These observations suggest that the SOSIP changes and the L193R or I423A change alters the Env conformation in distinct ways.

SOSIP changes stabilize gp120 in a State 2/3-prone conformation. To understand the individual contributions of the SOS and IP mutations to the increased recognition of the SOSIP L193R mutant by 17b (Fig. 3C), we introduced the SOS and I559P changes individually or in combination with L193R. The combination of L193R with I559P, but not with SOS, recapitulated the significant increase in 17b recognition observed with the SOSIP L193R mutant (Fig. 4). Thus, the I559P change in gp41 renders gp120 more prone to assume a State 2/3 conformation, which is ultimately achieved as a result of the L193R change. To support this contention, we evaluated the ability of the L193R change to promote gp41 HR1 exposure in response to sCD4. HR1 exposure was measured with the recombinant C34-Ig protein (33) and recapitulated the results obtained with 17b binding. While the L193R mutant was recognized by C34-Ig slightly better than its wild-type counterpart upon sCD4 addition, the combination of L193R with I559P resulted in an Env that exhibited significantly increased C34-Ig binding (Fig. 5). Similar results with C34-Ig were observed with the I423A mutant (not shown).

FIG 5 Effects of Env changes on gp41 HR1 exposure. 293T cells were transfected with an empty pcDNA3.1 plasmid or a plasmid expressing one of the indicated HIV-1_{BG505} Env variants. (A) Two days posttransfection, Env expression was evaluated by flow cytometry, as described in Materials and Methods, with antibody 2G12, which recognizes the gp120 outer domain. (B) HR1 exposure was assessed by flow cytometry, as described in Materials and Methods, with the Alexa Fluor 647-conjugated recombinant protein C34-Ig in the absence (gray histograms) or presence (black histograms) of 10 μ g/ml sCD4. (C) Recognition of Env variants in the presence or absence of sCD4 by Alexa Fluor 647-conjugated C34-Ig. Data are means and standard errors of the means. (D) Representative graph of sCD4-induced HR1 exposure, as measured by the binding of Alexa Fluor 647-conjugated C34-Ig by Env variants. Data represent the averages from five independent experiments, presented as the mean fluorescence intensity (MFI) detected with Alexa Fluor 647-conjugated C34-Ig divided by the MFI detected by antibody 2G12. Statistical significance was evaluated using an unpaired Student *t* test or a Mann-Whitney test (*, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; ns, not significant).

Impact of SOSIP, L193R, and I423A changes on CD4 engagement. CD4 engagement induces major conformational changes in Env that are required for virus entry. We reported previously that, relative to wild-type Env, CD4 recognition is decreased in Envs bearing the I559P change (27). In this study, we observed that the SOSIP changes decreased the interaction of HIV-1_{BG505} SOSIP Env with sCD4 (Fig. 3A). Therefore, we evaluated whether the SOSIP changes affect the potential cooperativity of sCD4 binding to Env. To do so, we incubated our panel of mutants with increasing concentrations of sCD4 and then revealed the presence of Env-bound sCD4 with the anti-CD4 antibody OKT4, which recognizes an epitope that does not overlap the gp120-binding site (34). These results were used to calculate the Hill coefficient of sCD4 interaction for each Env variant. The Hill coefficient reflects the degree of cooperativity between a ligand and its receptor (35). The wild-type Env exhibited a neutral Hill coefficient of ~ 0.9 . Introduction of the SOSIP changes resulted in a marked decrease in the Hill coefficient (to ~ 0.5); this negative cooperativity suggests that the interaction of sCD4 with one Env protomer reduces the efficiency with which additional sCD4 molecules engage the adjacent Env protomers (Fig. 6A). This phenotype of decreased sCD4 cooperativity was recapitulated for both the individual I559P (H, ~ 0.60) and SOS (H, ~ 0.61) changes (Fig. 6B and C). Thus, both components of SOSIP (i.e., the SOS and IP changes) can influence sCD4 cooperativity. While introduction of the State 2/3-stabilizing changes L193R and I423A enhanced sCD4 interaction at all concentrations tested (with maximal sCD4–2G12 binding of ~ 0.4 and ~ 0.6 for L193R and I423A,

FIG 6 Effects of Env changes on sCD4 engagement. The binding of sCD4 to HIV-1_{BG505} Env variants expressed on the cell surface was measured by a cell-based ELISA. Increasing concentrations of sCD4 (0 to 128 nM) were incubated with Env-expressing cells and were detected using an anti-CD4 antibody (OKT4), as described in Materials and Methods. Means and SEM derived from at least six independent experiments performed in quadruplicate are shown. The Hill coefficient was determined using GraphPad software.

respectively, in contrast to ~ 0.35 for wild-type Env), the L193R and I423A changes did not have a major impact on sCD4 cooperativity, as shown by Hill coefficients of ~ 0.98 and ~ 0.9 , respectively (Fig. 6D and E). The combination of L193R with the I559P, SOS, or SOSIP changes further enhanced sCD4 interaction (with maximal sCD4/2G12 binding of ~ 0.6 and ~ 1.0 for L193R- and I423A-bearing Envs, respectively). A combination of L193R with the I559P or SOS changes resulted in an increase in the Hill coefficient (to ~ 1.27 or ~ 1.4 , respectively), suggesting a slight increase in sCD4 cooperativity (Fig. 6F and G). However, introduction of the L193R change in conjunction with the SOSIP changes resulted in wild-type levels of cooperativity (H , ~ 0.99). Similarly, introduction of the I423A change into the I559P, SOS, or SOSIP mutant resulted in wild-type levels of cooperativity (Fig. 6I to K). Of note, the effects of all these changes on sCD4 cooperativity were specific for sCD4, since no major changes in the Hill coefficient were observed when the CD4-binding site antibody VRC01 was tested (Fig. 7).

We next evaluated the cooperativity of sCD4 binding to a soluble gp140 SOSIP.664 Env trimer. The ability of the HIV-1_{BG505} gp140 SOSIP.664 trimer to interact with sCD4 was evaluated by microscale thermophoresis, as described in Materials and Methods. The HIV-1_{BG505} gp140 SOSIP.664 trimer also exhibited negative cooperativity in binding sCD4, with a Hill coefficient of ~ 0.6 (Fig. 8A). Supporting this observation, isothermal titration calorimetry (ITC) experiments revealed that $\sim 60\%$ of the soluble gp140 SOSIP.664 trimer was able to engage sCD4 (Fig. 8C). This negative cooperativity was specific for sCD4 engagement, since full occupancy and a Hill coefficient of ~ 1 were observed for the VRC01 antibody (Fig. 8B and D). Overall, our data indicate that SOSIP changes have a significant detrimental effect on the engagement of sCD4 by membrane Env and soluble Envs truncated at position 664.

Impact of SOSIP, L193R, and I423A changes on the exposure of cluster A gp120 epitopes. One consequence of CD4 binding is the exposure of numerous CD4i epitopes. Several families of antibodies, including anti-CoRBS, anti-V1V2, anti-V3, anti-gp41, and anti-cluster A antibodies, recognize distinct groups of CD4i epitopes. Anti-cluster A antibodies recognize highly conserved epitopes located in the gp120 inner domain (36–41). Cluster A epitopes, such as that recognized by antibody A32, are occluded in the closed State 1 trimer but can be exposed upon the interaction of Env with membrane-bound CD4 (34). Interestingly, while CD4mc have been reported to

FIG 7 Effects of Env changes on VRC01 engagement. The binding of VRC01 to HIV-1_{BG505} Env variants expressed on the cell surface was measured by a cell-based ELISA. Increasing concentrations of VRC01 (0 to 32 nM) were incubated with Env-expressing cells and were detected as described in Materials and Methods. Means and SEM derived from at least six independent experiments performed in quadruplicate are shown. The Hill coefficient (H) was determined using Graph Pad software.

induce the formation of the gp120 bridging sheet and the CoRBS, they are unable to expose anti-cluster A epitopes on their own (42). We reported recently that CD4mc engagement in the Phe43 cavity induced only a partial opening of trimeric Env, sufficient to expose the CoRBS but not enough to expose inner domain cluster A epitopes (42). Interaction of CoRBS antibodies with two gp120 subunits within the CD4mc-sensitized trimer further opened the trimeric Env, exposing anti-cluster A epitopes (42). Here we explored whether the SOSIP changes affect the exposure of anti-cluster A epitopes. The wild-type HIV-1_{BG505} Env or Envs with the L193R, I559P, SOS, or SOSIP changes, alone or in combination, were expressed in 293T cells, which were incubated with an Alexa Fluor-conjugated A32 antibody. Incubation was performed in conjunction with the CD4mc BNM-III-170 in the presence or absence of 17b or its Fab1 fragment. As we reported recently (42), only the combination of CD4mc with a full CoRBS antibody, but not with its Fab1 fragment, enabled maximal A32 recognition of wild-type and L193R Envs (Fig. 9). Introduction of the I559P change alone or in the context of the SOSIP changes significantly decreased cluster A epitope exposure under all conditions tested. Although the L193R change was able to partially restore exposure of the A32 epitope for the I559P mutant, it failed to do so in the presence of the SOS changes. Thus, the SOS and I559P changes affect cluster A epitope exposure differently. Taken together, these results indicate that the SOSIP changes stabilize Env in a conformation that is unable to recapitulate all of the CD4-induced conformational changes negotiated by the native Env.

DISCUSSION

The unliganded HIV-1 Env trimer on the viral membrane can potentially sample three conformations: a metastable “closed” conformation (State 1), the “open” CD4-bound conformation (State 3), and an intermediate “partially open” conformation (State 2) (4, 5). Major research efforts are under way to gain insight into these different Env conformations, particularly State 1, a key target for entry inhibitors and vaccine-elicited antibodies. The introduction of an artificial disulfide bond linking the gp120 and gp41 subunits (17–20), in combination with the I559P change (20, 21) and a truncation beginning at gp41 residue 664, permitted structural characterization of soluble trimers

FIG 8 Interaction of the HIV-1_{BG505} sgp140 SOSIP.664 trimer with sCD4. (A and B) The binding of Alexa Fluor 647-labeled sCD4 (A) and VRC01 (B) to the HIV-1_{BG505} sgp140 SOSIP.664 Env trimer was measured by microscale thermophoresis. The Hill coefficient (H) is reported. (C and D) Titration of BG505 SOSIP.664 with sCD4 (C) and VRC01 (D) by isothermal titration calorimetry. Based on the stoichiometry of the titration data, only 50 to 60% of the HIV-1_{BG505} sgp140 SOSIP.664 trimer engages in binding sCD4 (C), whereas all three protomers of the trimer are functional with respect to VRC01 binding (D). The dashed lines indicate the molar ratio corresponding to half-saturation of the trimer, which is very close to the stoichiometry obtained from the analysis of the binding curves. Binding of sCD4 to the sgp140 SOSIP.664 trimer is characterized by a dissociation constant of 30 nM and changes in enthalpy (ΔH) and entropy ($-\Delta S$) of -22.7 and 12.2 kcal/mol, respectively. The dissociation constant for VRC01 is 52 nM, and the respective changes in enthalpy and entropy associated with the binding event are -29.9 and 20.0 kcal/mol. The concentration of trimer in the cell was about $1 \mu\text{M}$, and the concentration of sCD4 in the syringe was $40 \mu\text{M}$. The concentration of sCD4 in the syringe was $25 \mu\text{M}$, i.e., $50 \mu\text{M}$ Fab sites. The temperature was 25°C in all experiments.

in complex with different broadly neutralizing antibodies (bNAbs). The conformation sampled by these sgp140 SOSIP.664 trimers has been suggested to represent the “closed” native State 1 conformation (22–25, 29). To understand better the impact of SOSIP changes on Env conformation, we took advantage of well-established changes in gp120 (L193R and I423A) that shift the functional membrane-anchored Env to downstream States 2 and 3 (4, 16). We hypothesized that if Env bearing the SOSIP changes were stabilized in the State 1 conformation, then the introduction of the L193R or I423A changes should induce conformational changes similar to those in the wild-type Env

FIG 9 Impact of Env changes on anti-cluster A epitope exposure. 293T cells were transfected with an empty pcDNA3.1 plasmid or a plasmid expressing one of the indicated HIV-1_{BG505} Env variants. Two days posttransfection, Env expression was evaluated by recognition by MAb 2G12; the exposure of cluster A epitopes was assessed with the Alexa Fluor 647 (AF-647)-conjugated MAb A32 in the presence of the CoRBS antibody 17b or its Fab 1 fragment, with or without BNM-III-170, as described in Materials and Methods. (A) Data are presented as means and SEM of the mean fluorescence intensity (MFI) of A32 AF647 divided by the MFI detected by antibody 2G12. (B) Binding of MAb A32 to HIV-1_{BG505} Env variants in the presence of 17b and BNM-III-170. Data are averages from at least four independent experiments. Statistical significance was evaluated using the Kruskal-Wallis test or a Mann-Whitney test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant).

and should therefore shift Env into States 2/3. We performed our analysis using the membrane-anchored Env from HIV-1_{BG505}, the strain of origin of the soluble gp140 SOSIP.664 trimers used in most structural studies (22–24, 43, 44).

We first evaluated the impact of the SOSIP changes on Env conformation by comparing the antigenic profile of this mutant to that of its wild-type counterpart. In agreement with previous reports (27, 28), we found that introduction of the SOSIP changes decreased recognition by State 1-preferring ligands. Thus, the SOSIP changes stabilize the membrane Env in a conformation that differs from the wild-type State 1. When the SOSIP Env was compared to the State 2/3-stabilized L193R and I423A Env mutants, we also observed major differences in ligand recognition. Introduction of the L193R or I423A change in combination with the SOSIP changes resulted in conformations of the SOSIP Env that differed from those in the single L193R or I423A mutant. We observed that a combination of SOSIP and L193R or I423A changes resulted in recognition by some State 2/3-preferring ligands (17b, C34-Ig) at higher levels than those for the single L193R or I423A mutant. Taken together, these results suggest that SOSIP changes stabilize the membrane-anchored Env in a conformation that differs not only from State 1 but also from States 2/3, which represent functional default conformations for Envs with the State 1-destabilizing L193R or I423A change. The precise nature of the membrane Env conformation(s) stabilized by the SOSIP changes is unclear. For example, while it is known that the I559P change blocks Env function, it is not known whether this results from stabilization of a prefusion or an off-pathway conformation. In the soluble gp140 SOSIP Env constructs, further State 1-destabilizing influences likely result from loss of membrane association, leading to the default state seen in numerous crystal and cryo-electron microscopy structures.

We also explored the impact of the SOS and I559P changes on CD4 interaction. In marked contrast to the phenotypes of the wild type and its L193R and I423A variants, the I559P, SOS, and SOSIP mutants exhibited negative cooperativity for sCD4 binding. These results suggest that SOSIP-bearing Envs sample a conformation that differs from that of wild-type State 1 Env and interact with CD4 differently than native Env. Our studies emphasize the necessity to acquire structural information on the State 1 Env conformation, since this represents a key target for entry inhibitors and an important blueprint for vaccine design. Indeed, the elicitation of bNAbs, considered the gold-standard output of an efficient

HIV-1 vaccine, may require Env-based immunogens stabilized in State 1. Therefore, our results should motivate additional studies to define and stabilize this high-energy prefusion conformation. Gaining additional structural information on State 2 and State 3 Env conformations will also help to generate a more complete picture of the HIV-1 Env conformational landscape and Env-mediated viral entry.

MATERIALS AND METHODS

Cell lines. 293T human embryonic kidney cells, Cf2Th canine thymocytes (American Type Culture Collection), and HOS cells (NIH AIDS Research and Reference Reagent Program) were maintained at 37°C under 5% CO₂ in Dulbecco's modified Eagle's medium (Invitrogen) containing 5% fetal bovine serum (Sigma) and 100 µg/ml of penicillin-streptomycin (Mediatech). Cf2Th cells stably expressing human CD4 and CCR5 were grown in a medium supplemented with 0.4 mg/ml of G418 (Invitrogen) and 0.15 mg/ml of hygromycin B (Roche Diagnostics) (45).

Site-directed mutagenesis. The sequence of full-length clade A HIV-1_{BG505} Env (46) was codon optimized (GenScript) and cloned into the expression plasmid pcDNA3.1. An asparagine residue was introduced at position 332 to allow 2G12 recognition. Mutations were introduced individually or in combination into pcDNA3.1-BG505 N332 Env. Site-directed mutagenesis was performed using the QuikChange II XL site-directed mutagenesis protocol (Stratagene), and a stop codon was introduced to replace the codon for Gly 711, truncating the cytoplasmic tail (ΔCT) and enhancing cell surface expression of selected HIV-1_{BG505} Env variants. The presence of the desired mutations was determined by automated DNA sequencing. The numbering of the HIV-1 Env amino acid residues is based on that of the prototypic HXBc2 strain of HIV-1, where position 1 is the initial methionine (47).

Cell-based ELISA. Trimeric EnvΔCT at the surfaces of HOS cells was detected by cell-based ELISA, as described previously (27, 48, 49). Briefly, HOS cells were seeded in T-75 flasks (3 × 10⁶ cells per flask) and were transfected the next day with 22.5 µg of Env-expressing plasmids using the standard polyethyl- enimine (PEI; Polysciences, Inc., Warrington, PA, USA) transfection method. Twenty-four hours after transfection, cells were plated in 384-well plates (2 × 10⁴ cells per well). One day later, cells were washed twice with blocking buffer (10 mg/ml nonfat dry milk, 1.8 mM CaCl₂, 1 mM MgCl₂, 25 mM Tris [pH 7.5], and 140 mM NaCl) and then incubated for 1 h at room temperature with anti-HIV-1 Env monoclonal antibodies (MAbs) recognizing the coreceptor binding site (17b), CD4-binding site gp120 epitopes (VRC03, F105, and b12), gp120 V3 glycans (19b, GE2 JG8), the gp120 outer domain (2G12), the gp120–gp41 interface (PGT151), and gp41 cluster I (F240, 7B.2) epitopes. All ligands were diluted in blocking buffer. A horseradish peroxidase (HRP)-conjugated antibody specific for the Fc region of human or mouse IgG (Pierce) was then incubated with the samples for 45 min at room temperature. For all conditions, cells were washed 5 times with blocking buffer and 5 times with washing buffer. HRP enzyme activity was determined after the addition of 20 µl per well of a 1:1 mix of Western Lightning oxidizing and luminol reagents (PerkinElmer Life Sciences). Light emission was measured with an LB 941 TriStar luminometer (Berthold Technologies). To establish sCD4 Hill coefficients, cells were preincubated for 40 min at room temperature with increasing concentrations of sCD4 (0 to 128 nM) before detection with the anti-CD4 antibody OKT4 and analysis with Graph Pad software.

Recombinant luciferase viruses. Recombinant viruses containing the firefly luciferase gene were produced using calcium phosphate transfection of 293T cells with the HIV-1 proviral vector pNL4.3Env⁺-Luc and the pcDNA3.1 BG505 ΔCT plasmid expressing the wild-type or mutant HIV-1_{BG505} envelope glycoproteins at a ratio of 2:1. Two days after transfection, cell supernatants were harvested; the reverse transcriptase activities of all viruses were measured as described previously (50). The virus-containing supernatants were stored in aliquots at –80°C.

Infection by single-round luciferase viruses. Cf2Th-CD4/CCR5 target cells were seeded at a density of 5 × 10³/well in 96-well luminometer-compatible tissue culture plates (Corning) 24 h before infection. Normalized amounts of viruses (as evaluated by the reverse transcriptase activities of the viral stocks) were then added to the target cells, followed by spin infection at 800 × g for 1 h in 96-well plates at 25°C. Increasing concentrations of sCD4 or 17b were added to the viruses at least 1 h prior to the spin infection. In all cases, cells were incubated after the spin infection for 48 h at 37°C; the medium was then removed from each well, and the cells were lysed by the addition of 30 µl of passive lysis buffer (Promega) and three freeze-thaw cycles. An LB 941 TriStar luminometer (Berthold Technologies) was used to measure the luciferase activity of each well after the addition of 100 µl of luciferin buffer (15 mM MgSO₄, 15 mM KPO₄ [pH 7.8], 1 mM ATP, and 1 mM dithiothreitol) and 50 µl of 1 mM D-luciferin potassium salt (Prolume).

Flow cytometry. To assess the Env conformations of wild-type and mutant HIV-1_{BG505} Envs by flow cytometric analysis, 3 × 10⁵ 293T cells were transfected by the calcium phosphate method with the Env-expressing plasmids along with a pIRES-GFP vector, at a ratio of 2 µg of pcDNA3.1 BG505 ΔCT Env variants to 0.5 µg of green fluorescent protein (GFP). Sixteen hours posttransfection, cells were washed with fresh medium, and epitope exposure was evaluated 24 h later. The recombinant Alexa Fluor-conjugated C34-Ig protein (33, 51) was used to detect HR1 exposure, as described previously (52). Alternatively, transfected 293T cells were incubated with 17b (1 µg/ml) in the presence or absence of sCD4 (10 µg/ml) or 50 µM BNM-III-170 for 1 h at 37°C. Binding was assessed using a goat anti-human antibody coupled to Alexa Fluor 647 (Invitrogen). Antibody 2G12, recognizing the gp120 outer domain, was used to normalize Env expression on the cell surface.

To assess the exposure of the epitope recognized by the anti-cluster A antibody A32, transfected cells were incubated with 17b or its Fab 1 fragment (5 µg/ml) for 1 h at 37°C, alone or in combination with

50 μ M BNM-III-170. A32 epitope exposure was revealed by incubation with Alexa Fluor 647-conjugated A32. Antibody binding was detected by gating on GFP-positive cells with an LSR II cytometer (BD Biosciences, Mississauga, ON, Canada). Data analysis was performed using FlowJo, version X.0.7 (Tree Star, Ashland, OR, USA).

Expression and purification of soluble gp140 SOSIP.664 glycoproteins. The HIV-1_{BG505} soluble gp140 6R.SOSIP.664.T332N Env trimers with a C-terminal D7324 epitope tag (referred to below as soluble gp140 [sgp140] SOSIP.664 glycoproteins) were expressed by Effectene transfection of HOS cells with a plasmid provided by P. D. Kwong (Vaccine Research Center) (24). The HIV-1_{BG505} sgp140 SOSIP.664 trimers were secreted in the supernatants of HOS cells and were then purified by *Galanthus nivalis* lectin-affinity chromatography and size exclusion chromatography, using a Yarra 4000 column equilibrated with 20 mM Tris-HCl and 150 mM NaCl. The sgp140 SOSIP.664 glycoproteins were concentrated and stored at -80°C .

Microscale thermophoresis. Ligand binding and the cooperativity of binding to the HIV-1_{BG505} sgp140 SOSIP.664 trimers were analyzed by microscale thermophoresis on a Monolith NT.115 instrument (NanoTemper Technologies) (53). The ligands, sCD4 and antibody VRC01, were fluorescently labeled with Alexa Fluor 647 using a coupling kit (Thermo Fisher Scientific). The sgp140 SOSIP.664 Env was not modified. The fluorescently labeled sCD4 and antibody VRC01 were kept at a constant concentration of 1 nM. The unlabeled sgp140 SOSIP.664 Env was added at concentrations ranging from 210 pM to 6.9 μ M for sCD4 and from 15.2 pM to 500 nM for VRC01. The ligands and Env trimers were incubated at room temperature for 1 h. The samples were then loaded into Monolith capillary tubes, and the fluorescence distributions were measured over standard temperature shifts by use of a Monolith NT.115 instrument (NanoTemper Technologies) according to the manufacturer's recommendations. The changes in the fluorescent thermophoresis signal were plotted against the concentration of the serially diluted sgp140 SOSIP.664 Env. The K_d (dissociation constant) and Hill coefficient were determined using NanoTemper analysis software.

Isothermal calorimetry. The direct binding of sCD4 and MAb VRC01 to BG505 SOSIP.664 Env was studied at 25°C using a high-precision VP-ITC calorimeter from MicroCal/Malvern Instruments (Northampton, MA, USA). The titrations were performed by stepwise additions of 10- μ l aliquots of either CD4 or VRC01 to the calorimetric cell (~ 1.4 ml) containing the HIV-1_{BG505} sgp140 SOSIP.664 trimer. The concentration of trimer in the cell was about 1 μ M, and the concentration of soluble CD4 in the syringe was 40 μ M. The concentration of VRC01 in the syringe was 25 μ M, i.e., 50 μ M Fab sites. Prior to the experiments, all reagents were dialyzed against phosphate-buffered saline (PBS), pH 7.4 (Roche Diagnostics GmbH, Mannheim, Germany). The heat associated with binding was obtained by subtracting the heat of dilution from the heat of reaction. The individual heats were plotted as a function of the molar ratio, and nonlinear regression of the data provided the enthalpy change (ΔH), the association constant (K_a , calculated as $1/K_d$), and the stoichiometry associated with binding.

Statistical analysis. Statistics were analyzed using GraphPad Prism, version 7.0c (GraphPad, San Diego, CA, USA). Every data set was tested for statistical normality, and this information was used to apply the appropriate (parametric or nonparametric) statistical test, which is indicated in the relevant figure legend. P values of <0.05 were considered significant.

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We have no conflicts of interest to report.

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