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Release of gp120 Restraints Leads to an Entry-Competent Intermediate State of the HIV-1 Envelope Glycoproteins

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ABSTRACT Primary human immunodeficiency virus (HIV-1) envelope glycoprotein (Env) trimers [(gp120/gp41)3] typically exist in a metastable closed conformation (state 1). Binding the CD4 receptor triggers Env to undergo extensive conformational changes to mediate virus entry. We identified specific gp120 residues that restrain Env in state 1. Alteration of these restraining residues destabilized state 1, allowing Env to populate a functional conformation (state 2) intermediate between state 1 and the full CD4-bound state (state 3). Increased state 2 occupancy was associated with lower energy barriers between the states. State 2 was an obligate intermediate for all transitions between state 1 and state 3. State 2-enriched Env required lower CD4 concentrations to trigger virus entry and more efficiently infected cells expressing low levels of CD4. These Envs were resistant to several broadly neutralizing antibodies and small-molecule inhibitors. Thus, state 2 is an Env conformation on the virus entry pathway; sampling state 2 increases the adaptability of HIV-1 to different host cell receptor levels and immune environments. Our results provide new insights into the conformational regulation of HIV-1 entry.

IMPORTANCE The envelope glycoproteins (Env) of HIV-1 mediate virus entry and are the sole targets of neutralizing antibodies. Understanding the way that Env promotes HIV-1 entry can expedite drug and vaccine development. By destabilizing Env, we found that it assumes an intermediate state that is functional and obligate for transitions to entry-competent conformations. Increased sampling of this state enhances the ability of HIV-1 to infect cells that express low levels of the CD4 receptor and allows the virus to evade neutralizing antibodies and small-molecule inhibitors. These findings provide new mechanistic insights into the function and inhibition of HIV-1 Env and will contribute to ongoing therapeutic and prevention efforts to combat HIV-1.

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The entry of human immunodeficiency virus (HIV-1) into host cells is mediated by the viral envelope glycoprotein (Env) trimer. HIV-1 Env is composed of three gp120 exterior subunits noncovalently associated with three gp41 transmembrane subunits (1–3). Binding of gp120 to the CD4 receptor triggers the transition of Env from a metastable, high-energy state to downstream conformations. CD4-induced (CD4i) gp120 transitions include a repositioning of the V1/V2 and V3 loops and formation of the bridging sheet and coreceptor binding site (4–12). The heptad repeat (HR1) coiled coil in the gp41 ectodomain is also formed and exposed after CD4 binding (13–16). Subsequent binding to the CCR5 or CXCR4 coreceptor promotes the formation of a stable gp41 six-helix bundle, composed of the HR1 and HR2 heptad repeats, that mediates the fusion of the viral and target cell membranes (17–21).

The mature, unliganded Env of most primary clinical HIV-1 isolates assumes a “closed” conformation of the gp120 subunits at the trimmer apex (22–27). Here, we refer to the native “closed” Env conformation as state 1. CD4 binding rearranges the gp120 V1/V2 and V3 loops at the trimmer apex, thus “opening” the HIV-1 Env trimer to form the prehairpin intermediate (22), referred to here as state 3. Env transitions between state 1 and state 3 must be tightly regulated to allow entry into cells with different levels of receptor, while sequestering conserved Env elements from host neutralizing antibodies (NAbs). HIV-1 strains differ in the propensity of their Envs to make these transitions and sample downstream conformations; this property contributes to different requirements for target cell CD4 levels and different sensitivities to host neutralizing antibodies, small-molecule entry inhibitors, and incubation in the cold (28–37). Information on the entry-related transitions of HIV-1 Env can be obtained by identifying and studying functional conformational intermediates.

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Here, starting with the difficult-to-neutralize primary HIV-1JR-FL, we identify key residues in the gp120 V1/V2 elements that restrain Env movement from state 1 and thus regulate Env transitions to downstream conformations. Alteration of these restraining residues resulted in extensive conformational changes, generating entry-competent Env mutants. We then used selective bNAb’s to study the functional conservation of a restraining gp120 V2 residue among HIV-1 subtypes.

RESULTS

Entry-competent intermediate states of HIV-1 Env. We identified two groups of HIV-1 entry inhibitors and used them as chemical probes to study Env conformation: (i) CD4-mimetic compounds (CD4MCs) such as DMJ-II-121 and (ii) blockers of conformational change such as BMS-806 and 18A (16, 37, 38). CD4MCs and blockers of conformational change bind to different sites on HIV-1 gp120 (Fig. 1A) (38, 39). We found that these two groups of compounds exert opposing effects on structural rearrangements of Env (Fig. 1B). The CD4MC DMJ-II-121 induced the formation/exposure of the 17b epitope (near the coreceptor binding site of gp120 [40]) and the gp41 HR1 (recognized by the C34-Ig protein [16]). In contrast, the blocker of conformational change BMS-806 decreased 17b binding and blocked soluble CD4 (sCD4)-induced formation/exposure of gp41 HR1 (16). Thus, CD4MCs such as DMJ-II-121 induce an Env conformation similar to that induced by CD4 and therefore exhibit greater potency against Envs in the CD4-bound conformation (state 3). In contrast, blockers of conformational change such as BMS-806 and 18A demonstrate increased potency against Envs in state 1 (37).

We next used our chemical probes to identify specific amino acid changes that alter the conformation of the functional Env trimer on virions. On the basis of the observation that the HIV-1 gp120 V1/V2 and V3 regions contribute to contacts among the WT, L193A, and L193R HIV-1JR-FL viruses. smFRET analysis previously demonstrated that HIV-1 WT Env can sample at least three conformations detectable with FRET probes in the V1 and V4 variable regions of gp120 (43). Two conformations were defined with high confidence: state 1 (low FRET) represents the unliganded Env conformation, and state 3 (intermediate FRET) represents the full CD4-bound conformation. The identity and functional significance of the high-FRET state are currently unknown. This state was hypothesized to represent either a CD4-bound conformation or a previously uncharacterized and necessary structural intermediate (43). We defined state 2 as this high-FRET conformation and used the leucine 193 mutants to investigate its nature. The WT HIV-1JR-FL Env primarily occupied the "closed" conformation (state 1) and made infrequent transi-
Specific amino acid residues in the gp120 V1/V2 region regulate HIV-1 Env conformational transitions. (A) The binding sites of the small-molecule HIV-1 entry inhibitors used in this study are indicated by arrows on a ribbon structure of the gp120 core (PDB entry 1RZK). The gp120 core inner domain (purple), outer domain (magenta), and bridging sheet (orange) are shown. The location of the inhibitor binding sites is based on available crystal structures (38, 39). The gp120 residues contacting CD4 (12) are colored in cyan. (B) Flow cytometric analysis of the effect of the CD4-mimetic compound (CD4MC) (Continued)

FIG 1 Specific amino acid residues in the gp120 V1/V2 region regulate HIV-1 Env conformational transitions. (A) The binding sites of the small-molecule HIV-1 entry inhibitors used in this study are indicated by arrows on a ribbon structure of the gp120 core (PDB entry 1RZK). The gp120 core inner domain (purple), outer domain (magenta), and bridging sheet (orange) are shown. The location of the inhibitor binding sites is based on available crystal structures (38, 39). The gp120 residues contacting CD4 (12) are colored in cyan. (B) Flow cytometric analysis of the effect of the CD4-mimetic compound (CD4MC) (Continued)
itions from this state (Fig. 2A). Incubation with sCD4 moderately shifted some of the WT HIV-1JR-FL Env to downstream conformations (Fig. 2B). Notably, transitions of WT Env from state 1 to state 3 occurred exclusively through state 2, suggesting the potential functional importance of Env transitions through state 2. Compared with the WT Env, the unliganded L193 mutants exhibited a substantial shift in the occupancy of the conformational states. The occupancy of state 1 was lowered and the occupancy of state 2 was increased (with a smaller increase for state 3) for the unliganded L193 Envs relative to the WT Env (Fig. 2A, C, and E). Notably, the distribution of conformations of both L193 mutants in their unliganded state was similar to that of the WT Env incubated with sCD4. Transitions among the different conformations of the unliganded L193A and L193R Envs were more frequent than those detected for the WT Env, which was reflected in the higher rate constants calculated for transitions between conformational states of the L193 mutants (Fig. 2A, C, and E; see also Fig. S4 and Table S3 in the supplemental material). The higher rate constants indicate that the activation barriers separating the conformational states of the L193 mutants are lower than those of the WT Env. The magnitude of the increase in transitions of the L193A and L193R mutants was in agreement with their relative sensitivities to ligands recognizing downstream Env conformations. The addition of sCD4 to the L193 mutants resulted in profound transitions into downstream states, shifts that were significantly greater than that observed for the WT Env incubated with sCD4 (Fig. 2B, D, and F). CD4 binding to the L193A Env increased the occupancy of state 3; CD4 binding to the L193R Env increased the occupancies of both state 2 and state 3. As was observed for the WT Env, transitions of the L193A and L193R Envs from state 1 to state 3 occurred exclusively through state 2. Thus, alteration of the hydrophobic Leu 193 decreases the energy barriers between state 1 and downstream states, increasing the propensity of the mutant Env to sample state 2 and state 3, both spontaneously and after the binding of CD4 (Fig. 2G). These results relate functional Env phenotypes to conformational states defined by smFRET and illustrate the relationship between viral phenotypes and thermodynamic parameters. Because the Env L193 mutants retain the ability to bind receptors and mediate membrane fusion, our data support a model in which state 2 is a functional intermediate on the virus entry pathway.

**Enrichment of state 2 lowers the CD4 concentration required for HIV-1 entry.** Changes in Leu 192 in the V1/V2 loop of gp120 resulted in the highest increases in the sensitivities of the resulting virus variants to ligands recognizing downstream conformations. The inferred increase in Env sampling of downstream states is expected to enhance the ability of these HIV-1 variants to infect cells with lower levels of CD4 (28, 29, 31, 32). To evaluate the requirement of CD4 for infection, we incubated viruses displaying the WT and L193A Envs with CD4-negative, CCR5-expressing cells and measured the ability of different concentrations of the CD4MC DMI-II-121 or sCD4 to activate infection (Fig. 3A). Neither virus efficiently infected the cells in the absence of a CD4 mimic (data not shown). The L193A mutant required significantly less CD4MC or sCD4 to trigger virus entry than the WT Env, confirming its enhanced propensity to transition into the CD4-bound conformation.

As the ability to enter cells with low CD4 expression is one requirement for macrophage tropism (34, 44–47), we asked if the L193 mutants exhibited an increased ability to infect primary human macrophages compared with the wild-type virus. We generated wild-type and L193A and L193R mutant HIV-1JR-FL, normalized the virus titer according to infectivity on TZMbl cells, and infected human monocyte-derived macrophages. The ability of the two state-2-enriched mutants to infect primary macrophages was increased, on average, ~4-fold over that of the wild-type HIV-1JR-FL (Fig. 3B and C). A similar phenotype was observed for a recombinant HIV-1 with the L193A Env variant of HIV-1BG505, a non-macrophage-tropic strain (Fig. 3D and E). These data relate shifts in the energy landscapes of HIV-1 Env variants toward state 2 and state 3 to an increased ability of the virus to infect cells with low levels of CD4. Additional requirements shaped by the cellular and immunological environment ultimately determine HIV-1 macrophage tropism in vivo.

**Conformational preferences of broadly neutralizing antibodies.** The ability to modulate the energy landscape of the HIV-1JR-FL Env allowed us to evaluate the conformational preferences of antibodies generated in humans during HIV-1 infection. We examined the ability of polyclonal sera from two HIV-1-infected individuals to neutralize the panel of Leu 193 mutants. In both cases, the neutralization sensitivity of the mutants inversely correlated with the hydrophobicity of Leu 193 (Fig. 4A) (42). The sensitivity of the virus panel to the polyclonal sera correlated with the sensitivity to sCD4, 19b, 17b, and 902090 but not with the sensitivity to T20 (see Fig. S2 in the supplemental material). Thus, most of the neutralizing antibodies in these sera are directed against Env epitopes that are better exposed in state 2 than in state 1.

Broadly neutralizing antibodies (bNAbs) are elicited in only a minority of HIV-1-infected humans and after a long period of infection (48). We used the HIV-1JR-FL Leu 193 mutant panel to
evaluate bNAb selectivity for specific Env conformations. Three different patterns of conformational selectivity among these antibodies were identified. The first group included the CD4 binding site (CD4-BS) and V2 quaternary bNAbs and showed a strict preference for state 1. The WT Env was efficiently inhibited by these bNAbs; however, nonhydrophobic substitutions at residue 193 generated Env variants that were significantly more resistant to neutralization by these bNAbs (Fig. 4B to D; see also Fig. S5 in the supplemental material). In contrast, weakly neutralizing antibodies directed against related sites, namely, F105 (against the CD4-BS) and 830A (against the V2 β-barrel [49]), showed the opposite preference, inhibiting only viruses with Envs that moved from state 1 to downstream conformations. The preference of the CD4-BS and V2 quaternary bNAbs for state 1 is consistent with the occlusion or disruption of their respective epitopes by CD4 binding (37, 50, 51).

The second group of bNAbs included 35O22, which targets an Env epitope in the gp120-gp41 interface (52), and 10-1074, which targets a gp120 glycan-dependent V3 epitope (53). These bNAbs showed no preference for a specific Env conformation and equivalently neutralized viruses with Envs altered in gp120 residue 193 (Fig. 4E).

The third group of bNAbs included PGT151, which targets an Env epitope in the gp120-gp41 interface (54), and three bNAbs directed against the gp41 membrane-proximal external region (MPER). All these bNAbs exhibited a trend favoring a state 2 con-
Neutralization of HIV-1 variants differing in conformational state by human antibodies elicited during infection. (A) Recombinant viruses with Envs containing substitutions in residue 193 were tested for sensitivity to neutralization by antibodies. The relationship between the hydrophobicity of residue 193 and neutralization by antibodies is shown.

![Graphs showing neutralization by antibodies](FIG 4)

**C**

- **3BNC117**
  - $R_s = -0.87, P = 0.002$
  - Hydrophobicity vs. IC$_{50}$

- **VRC01**
  - $R_s = -0.59, P = 0.044$
  - Hydrophobicity vs. IC$_{50}$

- **VRC03**
  - $R_s = -0.90, P = 0.0001$
  - Hydrophobicity vs. IC$_{50}$

- **F105**
  - $R_s = 0.82, P = 0.002$
  - Hydrophobicity vs. IC$_{50}$

**D**

- **35O22**
  - $R_s = 0.27, P = 0.48$
  - Hydrophobicity vs. IC$_{50}$

- **PGT151**
  - $R_s = 0.79, P = 0.019$
  - Hydrophobicity vs. IC$_{50}$

- **10-1074**
  - $R_s = 0.48, P = 0.24$
  - Hydrophobicity vs. IC$_{50}$

**E**

- **10E8**
  - $R_s = 0.51, P = 0.12$
  - Hydrophobicity vs. IC$_{50}$

- **7H6**
  - $R_s = 0.58, P = 0.066$
  - Hydrophobicity vs. IC$_{50}$

- **4E10**
  - $R_s = 0.64, P = 0.029$
  - Hydrophobicity vs. IC$_{50}$

**F**

| Clade | State 2/3 preferred IC$_{50}$ | State 1 preferred IC$_{50}$ |
|-------|------------------------------|-----------------------------|
| Clade A | sCD4 4E10 | sCD4 4E10 |
| Clade B | sCD4 4E10 | sCD4 4E10 |
| Clade C | sCD4 4E10 | sCD4 4E10 |

(Continued)
formation, but only PGT151 and 4E10 antibodies showed a statistically significant preference (Fig. 4E). This observation is consistent with previous suggestions that some MPER epitopes on gp41 are more exposed after CD4 binding (55, 56). Overall, we found that many naturally elicited antibodies, including several bNAb, exhibit selectivity for specific Env conformations (see Table S4 in the supplemental material).

We next used the bNAb with high conformational selectivity to study the effects of the L193A change in the gp120 Env of other HIV-1 strains. The L193A change in HIV-1 strains from clades A, B, and C led to phenotypes consistent with those expected for state 2 or 3 (Fig. 4F). Ligands preferring downstream Env conformations (sCD4 and 4E10) inhibited the L193A viruses more effectively than the related WT viruses. Conversely, ligands preferring state 1 (PG9 and VRC03) more potently inhibited the WT strains. Thus, Env residue 193 maintains Env from different HIV-1 clades in state 1, countering transitions to downstream conformations. The high degree of conservation (98%) of leucine at this position among all HIV-1 strains is consistent with its key role in maintaining the integrity of state 1.

DISCUSSION

Our report provides new insights into the relationship between the Env conformational landscape, virus entry requirements, and HIV-1 susceptibility to inhibitors and antibodies. Changes in specific gp120 V1/V2 residues resulted in increased resistance to BMS-806, a blocker of conformational change, and increased sensitivity to sCD4, CD4MCs, and CD4i, V2, and V3 antibodies. Significant increases in sensitivity to T20, reflecting formation/exposure of the gp41 HR1 coiled coil, were not observed for most of these mutants. Moreover, the mutants remained CD4 dependent. Thus, these mutants sample a set of related entry-compatible conformations that are intermediate between state 1 and the full CD4-bound state (state 3). smFRET analysis revealed that these Env mutants are enriched in the occupancy of state 2, thus linking this obligate intermediate state to phenotypically characterized, functional Env conformations on the HIV-1 entry pathway.

The conformational states occupied by the functional HIV-1 Env trimer depend upon two related parameters: (i) the height of the activation barriers separating the states, which determines the rates of transitions between the states, and (ii) the relative free energies of the states, which dictate the occupancy of each state at equilibrium. The major fraction of primary HIV-1 Env resides in state 1, with transitions from this metastable state constrained by the high activation barriers separating state 1 and state 2 (Fig. 5). Under certain circumstances, these high activation barriers may allow Env-receptor engagement or antibody binding to state 1 before equilibrium among the available conformations is achieved. Indeed, antibodies that potently neutralize primary HIV-1 isolates exhibit high rates of binding to Env trimers in state 1 (41, 57, 58). The Env energy landscape changes upon CD4 binding and upon alteration of specific conformation-restricting residues in gp120 (Fig. 5). In both cases, the activation barriers and the differences in free energy between state 1 and downstream conformations are lowered, increasing the propensity for the CD4-bound and mutant HIV-1 Env to proceed along the entry pathway. Lowered activation barriers allow more transitions between states, and the smaller differences between the free energies of the states result in an increase in the occupancy of state 2 relative to that of state 1. Of note, multiple different Env residue changes resulted in qualitatively similar viral phenotypes; this observation favors a model in which changes in restraining Env residues destabilize state 1, allowing Env to proceed to state 2. Some of the Env changes may have fortuitously stabilized state 2 as well. State 2 encompasses a set of related conformations that reside in a local energy well. Previous studies suggested that the HIV-1 gp120 core has a propensity to sample the CD4-bound state when variable loop-mediated restraints are removed (59). In a similar manner, the more subtle losses of key molecular contacts resulting from single-residue changes in the gp120 trimer association domain release Env to assume one or more intermediate states. Importantly, Env in state 2 retain a high potential energy, which likely is required for Env function; however, due to the low activation barrier between states 2 and 3, Env in state 2 are more sensitive to triggering by ligands, including CD4 itself, that drive Env to the CD4-bound state. Destabilization of the ground state of a typical protein is expected to result in a decrease in function. In contrast, destabilization of state 1 creates increased opportunities for HIV-1 Env to sample downstream functional conformations. Smaller activation barriers and more-favorable free energy differences between state 1 and state 2 should allow a higher occupancy of Env protomers by CD4, lowering CD4 requirements for productive infection. CD4-independent HIV-1 may move even further along the entry pathway than the L193 mutants.

Alteration of several gp120 restraining residues, some of which exhibit variability in natural HIV-1 strains, can influence virus susceptibility to conformation-sensitive ligands. Hypersensitivity of the L193 Env mutants to these ligands reflects an increased sampling of state 2. We hypothesize that the observed variation in sensitivity of natural HIV-1 strains to conformation-sensitive ligands (32, 41, 60) results from altered activation barriers between state 1 and downstream conformations, either as a result of destabilization of state 1 or by changes more specific for the CD4 activation process. Viruses may thus achieve a balance between resis-

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Figure Legend Continued

sensitivity to neutralization by polyclonal sera (PS) from two HIV-1-infected individuals is shown. (B) Neutralization of WT and L193R HIV-1JR-FL by two different types of CD4-BS antibodies: the bNAb VRC03 and the weakly neutralizing F105 antibody. (C) Relationship between the hydrophobicity of Env residue 193 and HIV-1JR-FL sensitivity to neutralization by four CD4-BS antibodies: three bNAb (3BNC117, VRC01, and VRC03) and one weakly neutralizing antibody, F105. (D) Sensitivity of viruses with the indicated Env variants to neutralization by the PG9 antibody. The Env variants tested have changes distant from the defined V1/V2 binding site of the PG9 antibody (62). All these variants have, in addition, the E168K-plus-N188A changes that are required for the binding of PG9 to the HIV-1JR-FL Env (51). (E) The relationship between the hydrophobicity of Env residue 193 and HIV-1JR-FL sensitivity to neutralization by bNAb directed against gp120-gp41 hybrid epitopes (35O22 and PGT151), a V3 glycan-dependent epitope (10-1074), and gp41 MPER epitopes (10E8, 7H6, and 4E10) is shown. (A, C, and E) Spearman’s Rho coefficient and two-tailed P values are shown. (F) Conformation-selective bNAb and sCD4 were used to test the sensitivity of the WT and the L193A variant of HIV-1 strains BG505 (clade A), JR-FL (clade B), and ZM533.MPB12 (clade C). For PG9 neutralization, the E168K-N188A mutant of HIV-1JR-FL was used. Reported IC50 units are nanomolar (nM) for sCD4 and micrograms per milliliter (µg/ml) for the bNAb. Data shown are averages of results obtained in two or three independent experiments.
tance to neutralization by host antibodies and the level of target cell CD4 required to trigger virus entry.

MATERIALS AND METHODS
Detailed descriptions of the methods used are provided in Text S1 in the supplemental material.

Viral infection assay. A single-round infection assay was performed in 96-well plates by adding to each well a test compound or an antibody followed by supernatant containing a specific Env-pseudotyped virus (4 ng of p24) and then Cf2Th-CD4/CCR5 target cells. The activity of firefly luciferase, which was used as a reporter protein in the system, was measured after 48 h of incubation at 37°C. Values corresponding to 50% infectivity concentrations (IC50s) were calculated by fitting the data to the four-parameter (logistic) equation (37).

Single-molecule fluorescence resonance energy transfer. Analysis of the conformational dynamics of HIV-1 Env was done after enzymatic labeling of the V1 and V4 loops on native HIV-1 virions with Cy3 and Cy5 fluorophores, respectively (43).

Flow cytometry. Flow cytometric analysis was performed by incubating transfected 293T cells with various concentrations of a test compound or an antibody. Binding was detected with allophycocyanin-conjugated anti-human antibody and/or fluorescein isothiocyanate-conjugated anti-CD4 antibody and analyzed with a BD FACSCanto II flow cytometer (BD Biosciences).

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at mbio.asm.org lookup/suppl/doi:10.1128/mBio.01598-16/-/DCSupplemental.

Table S3, DOC file, 0.1 MB.
Table S4, DOC file, 0.1 MB.

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FIG 5 Model of HIV-1 Env conformational landscapes. Effects of time, CD4 binding, and alteration of restraining residues on the conformations occupied by a primary HIV-1 Env. Most primary HIV-1 Envs have a low propensity to change from state 1 (i.e., they have low Env reactivity [32]). Alteration of key restraining residues can convert an Env with low reactivity to a more reactive Env (large arrow). The conformational landscape of the unliganded high-reactivity Env resembles that of the CD4-bound low-reactivity Env. Small arrows indicate increased transition rates, relative to those of the unliganded low-reactivity Env.
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