Stable, uncleaved HIV-1 envelope glycoprotein gp140 forms a tightly folded trimer with a native-like structure

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The HIV-1 envelope spike [trimeric $(gp160)_3$, cleaved to $(gp120/gp41)_3$] is the mediator of viral entry and the principal target of humoral immune response to the virus. Production of a recombinant preparation that represents the functional spike poses a challenge for vaccine development, because the $(gp120/gp41)_3$ complex is prone to dissociation. We have reported previously that stable HIV-1 gp140 trimers, the uncleaved ectodomains of $(gp160)_3$, have nearly all of the antigenic properties expected for native viral spikes. Because of recent claims that uncleaved gp140 proteins may adopt a nonnative structure with three gp120 moieties "dangling" from a trimeric gp41 ectodomain in its postfusion conformation, we have inserted a long, flexible linker between gp120 and gp41 in our stable gp140 trimers to assess their stability and to analyze their conformation in solution. The modified trimer has biochemical and antigenic properties virtually identical to those of its unmodified counterpart. Both forms bind a single CD4 per trimer, suggesting that the trimeric conformation occludes two of the three CD4 sites even when a flexible linker has relieved the covalent constraint between gp120 and gp41. In contrast, an artificial trimer containing three gp120s flexibly tethered to a trimerization tag binds three CD4s and has antigenicity nearly identical to that of monomeric gp120. Moreover, the gp41 part of both modified and unmodified gp140 trimers has a structure very different from that of postfusion gp41. These results show that uncleaved gp140 trimers from suitable isolates have compact, native-like structures and support their use as candidate vaccine immunogens.

HIV-1 | envelope | gp140 | immunogen

The HIV-1 envelope glycoprotein mediates initial steps of virus infection by engaging cellular receptors and facilitating fusion of viral and target-cell membranes (1). Biosynthesis of the virus-encoded envelope glycoprotein yields a precursor, gp160, which following trimerization undergoes cleavage by a furin-like protease into two noncovalently associated fragments: the receptor-binding fragment, gp120, and the fusion fragment, gp41 (1). Three copies each of gp120 and gp41 form the mature envelope spikes $(gp120/gp41)_3$, the major viral surface antigen. Binding, through a site on gp120, to the host primary receptor, CD4, and then, through a second site, to a coreceptor (e.g., CCR5 or CXCR4) triggers large conformational changes that include reduced interaction between gp120 and gp41 (probably leading to dissociation of the former) and a cascade of ensuing gp41 refolding events (2, 3). Within the precursor gp160, gp41, with its C-terminal transmembrane (TM) segment anchored in the viral membrane, folds into a prefusion conformation. Cleavage of gp160 makes this prefusion conformation metastable with respect to a rearranged, postfusion conformation. Thus, the loss of constraint on gp41 that accompanies coreceptor binding to gp120 triggers a transition in gp41 to an extended, membrane-bridging conformation (sometimes called a "prehairpin" conformation) (4) with a hydrophobic "fusion peptide" at its N terminus inserted into the target-cell membrane and the TM segment in the viral membrane. This relatively long-lived, transient conformation is the target of fusion inhibitors, such as enfuvirtide (5), and of several broadly neutralizing antibodies (bnAbs) (6–8). Folding back of each chain into an α-helical hairpin creates a stable, six-helix bundle—the "postfusion conformation"—placing the fusion peptide and TM segment at the same end of the molecule. This irreversible refolding of gp41 brings the two membranes together, leading to bilayer fusion and viral entry. Thus, during the fusion process, there are at least three distinct conformational states of the envelope protein: the prefusion conformation of $(gp120/gp41)_3$, the extended intermediate of gp41, and the postfusion conformation of gp41 (with release of free gp120). Moreover, conformational changes of the prefusion form occur upon CD4 and perhaps also coreceptor binding.

The envelope glycoprotein is also the primary target of humoral responses in HIV-1–infected individuals. Studies of human monoclonal antibodies (mAbs) have identified a subset members that neutralize a wide range of HIV isolates (see Table S1 for a partial catalog and original references) (9, 10). These bnAbs are of particular interest, because they may guide a search for immunogens to elicit them in vaccinees. Epitopes on gp120 recognized by human bnAbs include the CD4-binding site, a trimer-specific epitope in the relatively invariant parts of the V2 and V3 loops, and a site near the base of the V3 loop involving an *N*-linked glycan at position 332. A glycan-dependent epitope spans both gp120 and gp41. The membrane-proximal external region (MPER) of gp41 binds a set of bnAbs that were among the earliest broad neutralizers discovered. The domain-swapped, dimeric

Significance

Many proposed strategies for HIV-1 vaccine development require use of a recombinant protein that mimics the native, functional HIV-1 envelope spike. The uncleaved ectodomain of trimeric $(gp160)_3$, gp140, has been used as a surrogate for the native state of the cleaved, viral spike. Because of recent claims that uncleaved gp140 adopts an irrelevant, nonnative structure, with three gp120s flexibly associated with a postfusion ("triggered") trimeric gp41, we have inserted a 20-residue, flexible linker between gp120 and gp41 into one of our previously characterized, stable, uncleaved gp140 trimers. We show that these trimers indeed form a compact, native-like structure with many antigenic properties expected for the functional envelope spike. Thus, they are promising envelope immunogens for inducing neutralizing antibody responses.

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antibody, 2G12, recognizes only glycans at defined positions, and its reactivity therefore depends on specific glycosylation patterns but not on many other aspects of the gp120 amino acid sequence.

Interesting groups of nonneutralizing antibodies, or with a very narrow range of isolates neutralized, include those that bind the so-called CD4-induced (CD4i) epitope, which overlaps the coreceptor site, on the bridging sheet of gp120, when the epitope becomes exposed by the conformational changes that accompany CD4 binding (Table S1). Nonneutralizing antibodies that interact with gp41 fall into two "clusters": those in cluster I, which recognize the "immunodominant" C-C loop of gp41, and those in cluster II, which bind strongly with a segment just preceding the MPER in the gp41 polypeptide chain, but only when gp41 is in the postfusion conformation. Most of the antibodies listed in Table S1 recognize conformation-dependent epitopes and thus are excellent molecular probes for defining the conformational state of the envelope trimer.

A form of gp140, stabilized by a disulfide crosslink between gp120 and gp41 (perhaps related to the disulfide between surface and TM subunits in many oncoretroviruses) was introduced over a decade ago (11) and subsequently modified by introducing an Ile-to-Pro mutation in gp41, to retard formation of the six-helix bundle (12). The product, known as SOSIP (SOS to designate the double cysteine mutations and IP to denote the isoleucine to proline change), is possible only with certain isolates, and the most widely studied has been BG505 SOSIP.664 (13–16). This modified gp140 trimer, which can be cleaved with furin without compromising stability, has greatly facilitated structural analysis, probably by eliminating large-scale conformational fluctuations (13, 14). In this variant, the MPER has been deleted, and the furin site has been replaced with a string of six arginines. Its structure, determined by both electron cryomicroscopy and X-ray crystallography, shows that (as expected from other fusion proteins) the conformation of gp41 in the prefusion state is distinct from the postfusion six-helix bundle (3, 17). The SOSIP.664 structure, which is an extremely important contribution to our understanding of envelope trimer molecular architecture, has some puzzling features. Docking CD4 onto the model suggests that it can bind all three gp120 sites with no clashes. This observation is at odds with a large body of evidence, including a recent biophysical study of the same trimer, showing that CD4 binding induces a substantial structural rearrangement (18–22). Moreover, uncleaved BG505 gp140 without the SOSIP modifications is unstable and heterogeneous. Can one really conclude, as suggested (14), that all HIV-1 uncleaved gp140 trimers are misfolded products resembling three gp120 moieties flexibly linked to a trimer gp41 in the postfusion, six-helix bundle conformation? The answer to this question is of considerable consequence, as it relates directly to design and production of candidate HIV-1 vaccine immunogens.

In the work reported here, we have inserted a flexible, 20-residue linker between gp120 and gp41 in the context of a previously characterized, stable gp140. The linker releases the tight covalent constraint between gp120 and gp41, and it should therefore mimic to some extent the furin cleavage (which is effectively a linker of infinite length). If an uncleaved gp140 trimer truly resembles "three balls on a string," as asserted (14), addition of a linker that can extend as much as 70 Å should exaggerate this property, causing the trimer with the inserted linker to have antigenic properties resembling three monomeric gp120s and a very large hydrodynamic radius. We find, to the contrary, that gp140 with the flexible linker, 20-residue insert (gp140–FL20) has antigenic properties essentially identical to those of the unmodified trimer and very different from those of free gp120 or of a construct with three gp120s held together by a heterologous trimerization tag. Moreover, the linker barely affects the hydrodynamic radius of the trimer, which is even slightly smaller than that of SOSIP. These results show that the stable, uncleaved gp140 trimers we have characterized previously are compact and native-like, and they support our suggestion that they are promising, envelope-based immunogens for clinical testing in vaccine development.

Results

Production of gp140–FL20 and gp120-fd. We have previously reported production in human cells of two stable and homogeneous gp140 trimer preparations derived from HIV-1 92UG037.8 (clade A) and C97ZA012 (clade C) (23). These envelope trimers have markedly different antigenic properties from those of their sequence-matched, monomeric gp120s, and they induce potent neutralizing antibody responses in guinea pigs with titers substantially higher than those elicited by the corresponding gp120 monomers (23). To address the concerns that these trimers might adopt a nonnative structure, with three gp120 moieties flexibly tethered to a trimeric gp41 ectodomain in a postfusion conformation, we have inserted a flexible, 20-residue linker between gp120 and gp41 in our 92UG037.8 gp140 trimer to create a new construct, designated gp140–FL20 (Fig. 1*A*). We imagine that this protein could in principle adopt two distinct conformations, as depicted in Fig. 1*B*. The protein was expressed in HEK 293T cells and purified following our established protocol (6, 23). It elutes as a single sharp peak from a sizing column, and SDS/PAGE stained by Coomassie shows no evident degradation or contamination (Fig. 2*A*). Both the gp140–FL20 and gp140 proteins have the same elution volume by gel filtration chromatography, suggesting that insertion of the flexible linker between gp120 and gp41 does not result in any substantial change in hydrodynamic radius of the trimer. The hydrodynamic radii of gp140 and gp140–FL20 trimers, measured by dynamic light scattering, are 7.6 and 7.9 nm, respectively (Fig. 1*C*), comparable to the radius reported for the BG505 SOSIP gp140 trimer (8.1 nm) (24). All three forms are therefore similarly compact in solution. Analytical ultracentrifugation (AUC) analysis confirms that gp140–FL20 is trimeric with a molecular mass of ~412 kDa (Table 1 and Fig. S1). Addition of the flexible linker between gp120 and gp41 allows for nearly complete in vitro processing by recombinant, soluble furin, consistent with better exposure of the cleavage site in gp140–FL20 than in unmodified gp140 (Fig. 2*A*, *Inset*). An artificially constructed gp120 trimer, gp120-fd, confirmed by AUC to have a molecular mass of ~380 kDa (Table 1 and Fig. S1),

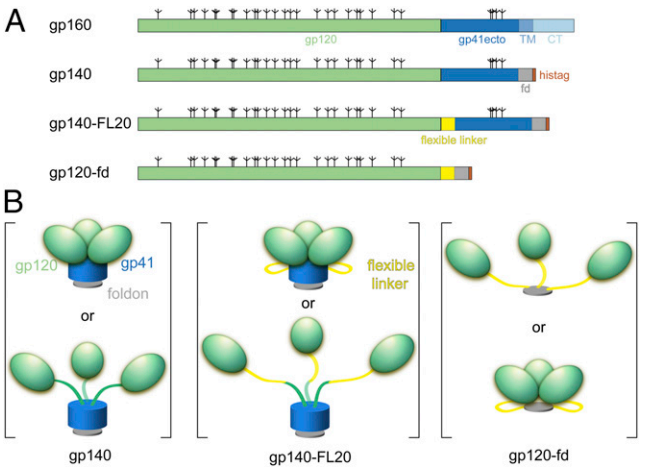

Fig. 1. Design of envelope expression constructs. (*A*) Schematic representations of HIV-1 envelope glycoproteins: gp160, the full-length precursor. Various segments include gp120, gp41 ectodomain (gp41ecto), TM anchor, and cytoplasmic tail (CT). Expression constructs are gp140, the uncleaved ectodomain of gp160 with a trimerization foldon tag and a His-tag at its C terminus; gp140–FL20, the uncleaved ectodomain of gp160 with a 20-residue linker $(SGGGG)_4$ inserted between gp120 and gp41, as well as a foldon tag and a His-tag at its C terminus; and gp120-fd, gp120 fused to a foldon tag and a His-tag through a 20-residue linker $(SGGGG)_4$. Tree-like symbols represent glycans. (*B*) Diagrams represent alternative 3D organizations of each protein species. Gp120 and gp41 are shown in green and blue, respectively. Foldon is in gray and the flexible linker in yellow. The conformation in the top row for each construct is supported by the data presented in this work.

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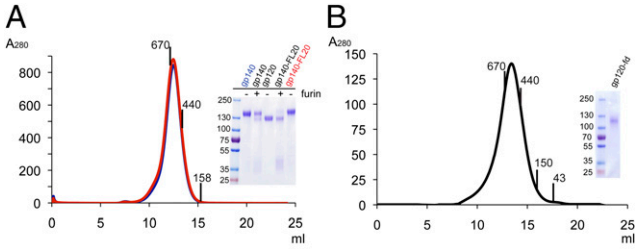


C

Dynamic light scattering analysis of the envelope proteins

Protein	Hydrodynamic radius (nm)	Polydispersity (nm)
gp 120	4.1	0.7
gp120-fd	9.0	5.0
gp140	7.6	1.7
gp140-FL20	7.9	1.9
BG505 SOSIP gp140[a]	8.1	n/a

Fig. 2. Production of gp140–FL20 and gp120-fd proteins in 293T cells. (*A*) His-tagged gp140 and gp140–FL20 proteins derived from the HIV-1 92UG037.8 were purified by Ni-NTA from supernatants of 293T cells transiently transfected with the expression constructs of these proteins. The purified gp140 (in black) and gp140–FL20 (in red) were resolved by gel filtration chromatography on a Superose 6 column. The molecular weight standards include thyoglobulin (670 kDa), ferritin (440 kDa), γ-globulin (158 kDa), and ovalbumin (44 kDa). Peak fractions were pooled and analyzed by Coomassie-stained SDS/PAGE (*Insets*). Purified gp140 and gp140–FL20 were also compared in a furin cleavage assay, and digested proteins were analyzed by SDS/PAGE as shown. (*B*) Purification of his-tagged gp120-fd. (*C*) Dynamic light scattering analysis of monomeric gp120, gp120-fd trimer, gp140 trimer, and gp140–FL20 trimer. [a]The hydrodynamic radius for the BG505 SOSIP gp140 trimer was reported in ref. 24.

with three copies of gp120 tethered to a foldon trimerization tag, elutes as a noticeably broader peak from a gel filtration column (Fig. 2*B*). The gp120-fd trimer thus appears to have a flexible structure (top row in Fig. 1*B*), sampling an ensemble of conformations with a wide range of hydrodynamic radii, as confirmed by dynamic light scattering analysis (Fig. 1*C*).

gp140–FL20 Distinguishes Between Neutralizing and Nonneutralizing CD4 Binding Site Antibodies. Our stable gp140 trimers bind only neutralizing CD4 binding site antibodies, such as VRC01 and CH31, but not nonneutralizing ones, such as b6 and 15e, whereas the monomeric gp120s derived from the same sequences react with all of them (6, 23). Insertion of a flexible linker between gp120 and gp41 could, in principle, set free the three gp120 moieties, if there were no substantial interactions between gp120 and gp41 or among gp120s and could expose all of the epitopes found on monomeric gp120. To test this hypothesis, we have used surface plasmon resonance (SPR) binding analysis to compare antigenic properties of the gp140, gp140–FL20, and gp120-fd trimers. To avoid potential artifacts, we immobilized an equal amount of each envelope trimer using an anti–His-tag antibody and then passed each Fab fragment of an HIV-directed antibody at various concentrations over the sensor chip surface. As shown in Fig. 3, all gp140, gp140–FL20, and gp120-fd trimers bound neutralizing, CD4 binding site antibodies VRC01, 12A12, and PGV04 almost equally well, with affinities ranging from 1.2 to 27 nM (Table S2), whereas preincubation of soluble CD4 with these trimers completely blocked these interactions. We have reported previously that a 92UG037.8 gp140 trimer binds only a single CD4 (23). Thus, one CD4 molecule and/or conformational changes induced by one CD4 can block all three CD4 binding sites on a gp140 trimer that are otherwise accessible to VRC01 Fab (23). Both gp140 and gp140–FL20 trimers show no binding at all to the nonneutralizing antibody b6, even in the presence of soluble CD4 (again one CD4 per trimer), suggesting that the trimer organization masks the nonneutralizing CD4 binding site epitope, as expected for the native functional envelope spikes on the surface of virion, and that CD4 binding does not open up the trimer. In contrast, b6 reacts very strongly with the gp120-fd trimer, with a dissociation constant of 3.2 nM (Table S2), in agreement with the antigenicity of the monomeric gp120, indicating that the three gp120s of gp120-fd do not associate into a native-like trimer structure, despite being brought together by a trimerization domain (depicted in Fig. 1*B*). These results demonstrate that the stable 92UG037.8 gp140 trimers have a compact, native-like structure, distinct from the one adopted by gp120-fd, which resembles three balls on a string.

gp120 in gp140–FL20 Has a Conformation Different from the CD4-Bound State. CD4i antibodies 17b and 412d detect formation of the bridging sheet in gp120 induced by CD4 binding. The gp140–FL20 and gp140 trimers have identical properties with respect to these antibodies (Fig. 3). They both show negligible binding with either 17b or 412D, even at high Fab concentrations, in the absence of CD4, but they bind the two antibodies tightly in the presence of soluble CD4, with dissociation constants of 3.2–38.0 nM (Table S2). Like monomeric 92UG037.8 gp120 (23), the trimeric gp120-fd protein binds 17b, but not 412d, in the absence of CD4, with a dissociation constant of 21 nM. Upon binding to CD4, gp120-fd shows much stronger interactions with both 17b and 412d, as expected (Fig. 3 and Table S2). The response units for binding the two antibodies to gp120-fd are substantially greater than those for the gp140 trimers when CD4 is present, although the number of binding sites available on all of the sensor surfaces should be the same. The differences can probably be explained by differences between the gp120-fd and gp140 trimers' stoichiometry of binding with these antibodies (see *gp140–FL20 Trimer Binds a Single CD4*). These observations demonstrate that the gp140 trimers have a conformation distinct from the CD4-triggered state and also different from the free monomeric gp120 structure.

gp140–FL20 Trimer Binds a Single CD4. The flexible linker between gp120 and gp41 could, in principle, change the ratio of CD4 to trimer from 1:1–3:1, if the three gp120 moieties of the gp140–FL20 trimer were only loosely attached to the trimeric gp41 ectodomain, as others have claimed (14). We determined by AUC the molecular masses of complexes containing gp120-fd or gp140–FL20 with two-domain CD4, VRC01, and 412d (Table 1). The masses of unliganded gp140–FL20 and gp120-fd were ∼411 and 380 kDa, respectively, and those of the complexes with two-domain CD4 were ∼438 and 443 kDa, showing that gp140–FL20 has only one bound CD4 molecule per trimer but that gp120-fd has three. As controls, the masses determined for the VRC01 Fab–gp140–FL20 and VRC01 Fab–gp120-fd complex were ∼532 and 520 kDa, respectively, consistent with a ratio of 3:1 for both trimers. In addition, the measured molecular mass of the gp140–FL20–CD4–412d

Table 1. Molecular mass determination by AUC and MALS

Protein	Molecular mass (kDa) by AUC	Molecular mass (kDa) by MALS
gp140–FL20*	411 ± 8	422 ± 1
gp140–FL20 + CD4[†]	438 ± 6 (2D CD4)	464 ± 3 (4D CD4)
gp140–FL20 + VRC01 Fab	532 ± 9	548 ± 3
gp140–FL20 + CD4 + 412d (or 17b) Fab[‡]	480 ± 11 (2D CD4)	504 ± 5 (4D CD4)
gp120-fd	380 ± 15	375 ± 9
gp120-fd + CD4	443 ± 8 (2D CD4)	511 ± 6 (4D CD4)
gp120-fd + VRC01 Fab	520 ± 12	520 ± 10
gp120-fd + CD4 + 17b Fab	n.d.[g]	644 ± 5 (4D CD4)
4D CD4	n.d.	46 ± 8
VRC01 Fab	n.d.	52 ± 4
17b Fab	n.d.	51 ± 6

AUC, analytical ultracentrifugation; MALS, multiangle light scattering; n.d., not determined. Detailed data analyses are shown in Figs. S1 and S2.
*All envelope constructs are derived from the HIV-1 92UG037.8 sequence.
[†]CD4, 2 domain soluble CD4 was used for AUC and 4 domain CD4 used for MALS.
[‡]412d was used for AUC and 17b was used for MALS.

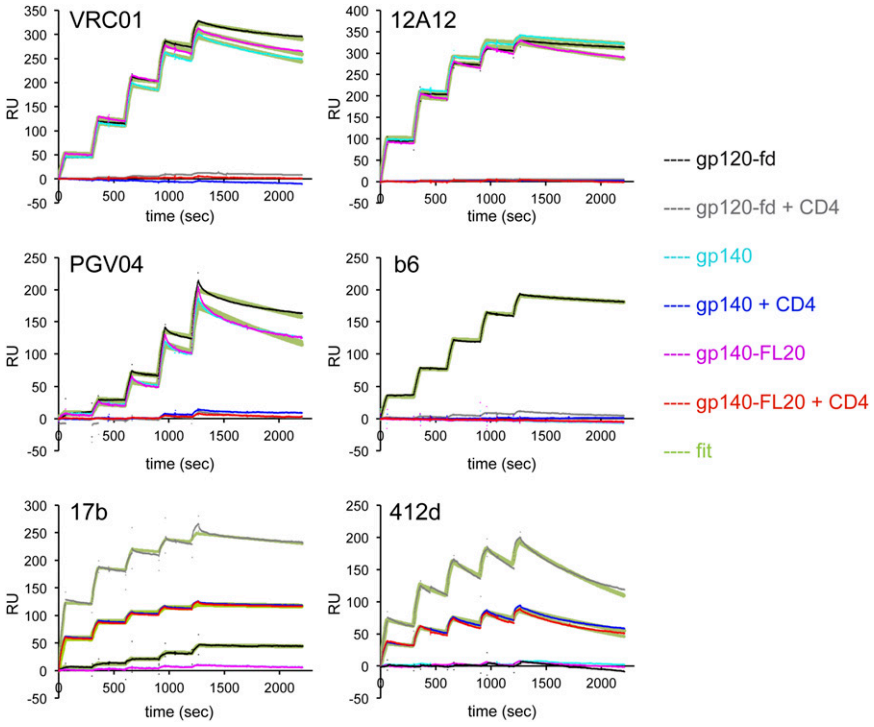


Fig. 3. Interactions of gp140, gp140–FL20, and gp120-fd with CD4 binding site and CD4i antibodies. Envelope proteins or their purified complexes with two-domain CD4 were captured to the surface of a sensor chip coated with an anti-HIS antibody to avoid potential artifacts introduced by protein immobilization. CD4 binding site antibodies include bnAbs VRC01, 12A12, PGV04, and a nonneutralizing antibody b6. CD4i antibodies are 17b and 412d. Fab fragment of each antibody at various concentrations were passed over the envelope trimer surface individually without regeneration for single-cycle kinetic analysis. The recorded sensorgram for gp140 is in cyan, gp140–CD4 complex in blue, gp140–FL20 in magenta, gp140–FL20–CD4 complex in red, gp120-fd in black, and gp120-fd–CD4 complex in gray; the fitted curves are in green. Sensorgrams were fit using a 1:1 binding model; and binding constructs are summarized in Table S2.

complex was ~480 kDa, indicating that one CD4-bound gp140–FL20 trimer binds only one 412d Fab. Multiangle light scattering (MALS) analysis using four-domain CD4 yields the same result (Table 1 and Fig. S2). These findings confirm that our gp140 and gp140–FL20 trimers assume a tightly packed, native-like conformation with strong noncovalent interactions among three gp120s, and presumably with gp41 as well, and that even CD4 binding cannot open up the trimer or induce gp120 dissociation.

V2 and V3 Epitopes are Similarly Accessible in both gp140 and gp140–FL20 Trimers. We also examined exposure of the V2 and V3 epitopes in the gp140 trimers using two V3-directed antibodies, 3791 and 19b, and a V2-directed antibody, 2158 (25–27). Antibodies 3791 and 19b recognize a neutralizing epitope located on the side of the V3 β-hairpin and on the tip ("crown") of the V3 loop, respectively (25, 27). As shown in Fig. S3, 3791 binds all three envelope trimers tightly either in the absence or presence of soluble CD4, with affinities ranging from 2.6 to 11.9 nM (Table S2), suggesting that its epitope is well exposed on all of the proteins. Upon CD4 binding, slightly better exposure of the 3791 epitope is observed only in gp140–FL20 trimer, but not in gp140 or gp120-fd trimers. Likewise, the epitope of 19b is equally accessible in all three trimers, with or without soluble CD4, despite much weaker binding than 3791 to gp120 with the 92UG037.8 sequence. The 2158 epitope involves the *N*-linked glycan at residue 186 and the singular face of the V2 loop (26). This epitope also appears to be accessible on all three trimers; it appears to be most exposed on gp120-fd (K_d = 2.2 nM) and least exposed on gp140–FL20 (K_d = 10 nM), but the differences are small. When CD4 binds, the epitope becomes somewhat less accessible on all three trimers. Overall, the V2 and V3 epitopes are well exposed on gp140 trimers, and CD4 binding has only a small impact on their exposure.

Glycan-Dependent, Broadly Neutralizing Epitopes. We also assessed binding of our gp140 trimers to three bnAbs that recognize *N*-linked glycan-dependent epitopes, including 2G12, PGT128, and PG9 (Fig. S4). In general, gp140 and gp140–FL20 trimers behave almost identically in these binding assays regardless of whether soluble CD4 is present or not, whereas gp120-fd trimer shows slightly but significantly better binding to 2G12 and PGT128, but no binding to PG9 at all, suggesting that the relevant epitopes on gp140 and gp140–FL20 have identical structures, different from their structure on the gp120-fd trimer. We also note that PG9 binding to the two gp140 trimers is completely lost when CD4 is present, indicating that this quaternary epitope is disrupted by CD4-induced structural rearrangements.

The gp41 Ectodomain in the 92UG037.8 gp140 Trimer Is Not in a "Triggered" Conformation. MPER-directed bnAbs 2F5 and 4E10 target the gp41 prehairpin intermediate and bind strongly with gp41-inter, a construct designed to mimic the extended intermediate conformation (6) (Fig. S5). Like our other gp140 trimers, gp140–FL20 does not bind 2F5 and 4E10 at all (Fig. S5), consistent with the properties of native functional envelope trimers expressed on viral or cell surfaces (7). Both the gp140 and gp140–FL20 trimers show detectable binding of nonneutralizing cluster I antibodies 240-D and 7b2, with dissociation constants ranging from 313 to 670 nM, but the affinity of the trimers for these antibodies is much weaker than that of gp41-inter, in which the epitopes are optimally exposed (6). These results indicate that the C-C loop of gp41 is not fully accessible in the gp140 and gp140–FL20 trimers and that insertion of a flexible linker between gp120 and gp41—and perhaps even cleavage—has no detectable impact on exposure of the cluster I epitope. Another nonneutralizing antibody, 1281, which targets the cluster II epitope when gp41 is in its postfusion conformation, has similar properties (28). We detected binding of cluster I and II antibodies (K_d < 500 nM) by the gp140 and gp140–FL20 trimers, with or without soluble CD4, but their affinity for the trimers was an order of magnitude lower than for a gp41 construct containing the six-helix bundle (Fig. S5). These results confirm that the gp41 ectodomain in the gp140 trimers, with or without the linker, is not in either of the CD4-induced, triggered conformations—the prehairpin-intermediate or the postfusion six-helix bundle.

Discussion

We have shown in the experiments presented here that stable, homogenous, uncleaved gp140 trimers have nearly all of the antigenic properties expected for a native-like, functional envelope trimer. Insertion of a 20-residue flexible linker to relax the covalent

constraints between gp120 and gp41 has minimal impact on compactness and antigenicity. These trimers are better immunogens than monomeric gp120 (29) and suitable for large-scale GMP productions (23, 30). Their simple design, from native sequences with no internal modification, makes these stable, native-like trimers attractive candidates for testing in human vaccine trials.

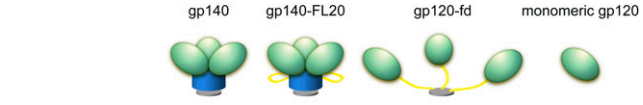
Induction of high levels of broadly reactive neutralizing antibody responses against HIV-1 is widely considered an important goal for vaccine development. Most bnAbs target the functional envelope trimer. It has been suggested that a recombinant form that represents the cleaved trimer might be necessary to elicit such antibodies. One such attempt, the BG505 SOSIP gp140 trimer, involves replacing the native furin cleavage site between gp120 and gp41 with a poly Arg sequence to enhance the cleavage efficiency, introducing an artificial disulfide bond between gp120 and gp41, mutating an isoleucine to a proline in gp41 to stabilize the cleaved trimer (by inhibiting helix formation in the corresponding segment), and removing the hydrophobic MPER to increase solubility (13, 14, 31). These modifications have been exceptionally useful for creating a trimer amenable to structural studies (15, 32), but it remains unclear whether it accurately represents the native, functional envelope trimer and, if it does, whether it is the best way to do so. Indeed, the structures of the BG505 SOSIP gp140 trimer, determined by crystallography at 3.5 Å (33) and by cryo-EM at 5.8 Å, respectively, show no clashes with CD4 when superposed on the structure of a gp120–CD4 complex (Fig. S6). This absence of interference is puzzling considering the well-documented structural rearrangements in gp120 that accompany CD4 binding (18–22). In some of the experiments reported here, we find that CD4 binding not only induces formation or exposure of the epitopes for 17b and 412d but also disrupts the quaternary epitope targeted by PG9.

Our gp140 and gp140–FL20 trimers show nearly identical biochemical and antigenic properties. In solution, they are at least as compact as the SOSIP trimer, as reported by dynamic light scattering. Despite suggestions that the cleavage between gp120 and gp41 may lead to large structural rearrangements of the envelope trimer and sequestration of nonneutralizing epitopes (14, 34), our stable gp140 already has minimal exposure of such epitopes. Moreover, insertion of a flexible linker, long enough to allow gp120 to dissociate from gp41, does not lead to any obvious global changes in antigenic surfaces on the trimer. This observation argues against the notion that the cleavage may rearrange the trimer substantially. We note that cleavage between HA_1 and HA_2 of influenza hemagglutinin allows a local (and functionally important) conformational rearrangement of the fusion peptide but leads to no large structural or antigenic changes (35). Connecting the gp120 and gp41 ectodomains by flexible linkers has been reported previously to yield "tethered Envs," which were thought to capture a transient conformation exposing conserved gp41 structures. These constructs potently inhibited membrane fusion mediated by a variety of HIV-1 isolates (36). In our gp140–FL20 construct, the C terminus of the gp41 ectodomain is locked by a trimerization tag, which mimics constraints from the viral membrane, and the gp41 trimer cannot undergo the structural changes that would lead to its postfusion conformation. Indeed, neither our gp140 nor gp140–FL20 trimer inhibits membrane fusion at concentrations (<10 μg/mL) tested for the tethered Envs in a cell–cell fusion assay, whereas both gp140 and gp140–FL20 block cell–cell fusion as efficiently as does monomeric gp120 at high concentration (100 μg/mL), presumably by competing for CD4 binding (Table S3). One study concluded that the gp41 part of our gp140 trimers adopts a postfusion six-helix bundle structure devoid of specific interactions between gp120 and gp41. That conclusion, which was based on protease resistance and on hydrogen/deuterium-exchange mass spectrometry data (37), is flawed, because we expect gp41 in the native-like trimer to be equally protease resistant and poorly exchanging. If the authors of that study had been correct about our gp140 trimers, the gp140–FL20 protein in our present experiments should have behaved like the gp120-fd trimer, and the gp140 trimers should have reacted strongly with six-helix bundle-specific antibodies, such as 1281 (28). Moreover, the properties of our gp120-fd trimer show that three gp120s would not come together to form a native-like trimer when held together only by a trimerization tag.

It is not possible to verify whether a particular soluble gp140 trimer preparation truly represents the functional envelope spike, as it lacks the essential TM segment and therefore cannot induce membrane fusion under any circumstances. Binding to neutralizing antibodies and lack of binding to nonneutralizing antibodies are useful guides if we compare these properties to those of the virion. In this context, it is important to remember that some the bnAbs, such as 2F5, 4E10, and 10E8, recognize a triggered form of the envelope trimer, not the native, prefusion conformation.

The stoichiometry of CD4 (1:1) and CD4i antibody (1:1:1) binding, observed previously for stable 92UG037.8 gp140 trimers, is also the case with gp140–FL20, in which the three gp120 moieties have potentially more freedom to move away from gp41 and from each other. CD4-liganded spikes on the surface of virions (presumably fully cleaved) appear, when visualized by electron cryotomography, to have three bound CD4 molecules. The virus used in that work, 2,2′-dithiodipyridine–inactivated HIV-1 BaL, binds 17b even in the absence of CD4, and its envelope protein may spontaneously adopt a CD4-bound conformation much more readily than patient isolates such as 92UG037.8 (38). The strong negative cooperativity of CD4 binding by stable 92UG037.8 trimers may be influenced by the covalent linkage between gp120 and gp41, by constraints from the C-terminal foldon tag, or by both. BG505 SOSIP has neither of these constraints, which could influence CD4 association by preventing a concerted conformational change. The cleaved BG505 SOSIP gp140 trimer, which lacks the MPER and has no C-terminal foldon, binds three CD4 molecules and three 17b Fabs (13). Nonetheless, both 92UG037.8 gp140 (or gp140–FL20) and BG505 SOSIP bind the same number of Fabs, for each type we have studied, with similar affinities. Sorting out the reason for the unexpected stoichiometry of CD4 binding to uncleaved gp140 and its potential physiological relevance will require further experiments.

We expect that native Env spikes—and recombinant products that accurately represent them—are in a dynamic equilibrium between conformations that occlude the receptor binding site and those that expose it. CD4 binds selectively to trimers in the latter state and stabilizes that bound conformation. The mutations that often produce sensitivity to CD4 neutralization in laboratory passaged strains of the virus shift the equilibrium, with complete CD4 independence as an extreme property (29, 39–41). The modifications introduced to generate SOSIP fix gp140 in a particular conformation, making that form especially favorable for structural studies by electron microscopy (both negative stain and cryo) and



	gp140	gp140-FL20	gp120-fd	monomeric gp120
non-neutralizing CD4 binding site antibody binding	NO	NO	YES	YES
CD4i antibody binding in absence of CD4	NO	NO	YES	YES
stoichiometry of CD4 binding (env:CD4)	1:1	1:1	1:3	1:1
stoichiometry of CD4i antibody binding in presence of CD4 (env:CD4:CD4i ab)	1:1:1	1:1:1	1:3:3	1:1:1
hydrodynamic radius (nm)/ molecular mass (kDa)	7.6/411	7.9/438	9.0/380	4.1/130

Fig. 4. Summary of the key differences between the gp140 and gp120 constructs. Molecular mass of the monomeric 92UG037.8 gp120 was reported previously in ref. 23. As a comparison, the hydrodynamic radius and molecular mass for the BG505 SOSIP gp140 trimer are 8.1 nm and 365 kDa, respectively (24, 44).

X-ray crystallography. In negative stain, less constrained trimers will tend to flatten and distort, as observed with essentially all molecular complexes of this size when negatively stained on carbon supports (42). Thus, one cannot use apparent uniformity (or nonuniformity) of images from negative staining as a sole criterion of conformational homogeneity or compactness, and we regard the hydrodynamic properties of 92UG037.8 gp140 as more reliable indicators of a compact and uniform conformation in solution.

The SOSIP gp140 is the only cleaved but nondissociating preparation of the envelope trimer, but large-scale production for clinical use presents a number of challenges (43). Moreover, for immunogenicity in animal models, it remains unclear whether there are real benefits of this artificially stabilized trimer over other envelope trimer immunogens. Indeed, an overly stabilized protein may not be the best immunogen for inducing neutralizing antibodies that need to recognize a functional target with intrinsic flexibility. We conclude that our data, as summarized in Fig. 4, warrant use of stable, uncleaved gp140 trimers with a native-like structure in human vaccine trials.

Materials and Methods

HIV-1 envelope proteins and soluble CD4 were produced in 293T cells. mAbs were produced in either 293T cells or hybridomas except for b6, which was expressed in CHO cells. Protein production, furin cleavage analysis, SPR binding assays, dynamic light scattering analysis, AUC, MALS, and inhibition of cell–cell fusion by envelope proteins were carried out following the detailed protocols described in *SI Materials and Methods*.

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