Prefusion structure of trimeric HIV-1 envelope glycoprotein determined by cryo-electron microscopy

Alberto Bartesaghi, Alan Merk, Mario J Borgnia, Jacqueline L S Milne & Sriram Subramaniam

The activation of trimeric HIV-1 envelope glycoprotein (Env) by its binding to the cell-surface receptor CD4 and co-receptors (CCR5 or CXCR4) represents the first of a series of events that lead to fusion between viral and target-cell membranes. Here, we present the cryo-EM structure, at subnanometer resolution (~6 Å at 0.143 FSC), of the ‘closed’, prefusion state of trimeric HIV-1 Env complexed to the broadly neutralizing antibody VRC03. We show that three gp41 helices at the core of the trimer serve as an anchor around which the rest of Env is reorganized upon activation to the ‘open’ quaternary conformation. The architecture of trimeric HIV-1 Env in the prefusion state and in the activated intermediate state resembles the corresponding states of influenza hemagglutinin trimers, thus providing direct evidence for the similarity in entry mechanisms used by HIV-1, influenza and related enveloped viruses.

Structural information on the trimeric envelope glycoprotein (Env), the only HIV-1 protein displayed on the surface of the viral membrane, is critical for rational vaccine design and for understanding of the detailed mechanisms of viral entry. Env is a heterodimer of a transmembrane glycoprotein (gp120) and a surface glycoprotein (gp41); these dimers are organized as trimers on the surface of the viral membrane. Structural studies of Env have been carried out over the past two decades with a variety of complementary structural methodologies, using preparations ranging from truncated variants of gp120 or gp41 to intact, native trimers. After solution of the first crystallographic structure of truncated monomeric gp120 in complex with soluble CD4 and the Fab fragment of the monoclonal antibody 17b, numerous crystal structures of the core fragment of gp120 with and without bound ligands were reported. The conformation of gp120 in all those structures is similar, irrespective of the presence or absence of bound ligands. Numerous crystal structures of the six-helix bundle formed by gp41 in the postfusion state are also available.

At the other end of the spectrum, cryo-electron tomographic methods, used in conjunction with newly developed tools for subvolume averaging, have enabled determination of several structures of the entire HIV-1–gp120–gp41 trimer, as displayed on intact viruses. On the basis of these studies, we showed that when trimeric Env is in the ligand-free state, or when it is bound to broadly neutralizing antibodies directed to the CD4–binding site (VRC01, VRC02 or VRC03), it is in a closed quaternary conformation with the V1V2 loop located close to the apex of the spike. When native trimeric HIV-1 Env is bound to CD4, or to co-receptor mimics such as antibodies 17b or m36, it transitions to an open state. The transition requires a large movement of each gp120 protomer, and relocates the V1V2 loop to the periphery of the trimer. These analyses of native HIV-1 Env delineate the closed and open quaternary conformations and reveal the activation of the trimer after its contact with cell-surface receptors, thus defining a key transition in the structural landscape of Env that is relevant to initial steps in viral entry.

In addition to analyses of the HIV-1 Env structure with native, membrane-bound trimeric HIV-1 Env, we have also extended these studies to soluble variants of trimeric Env. The ectodomain of HIV-1 Env is a heterodimer, with a mass of ~140 kDa, composed of the entire gp120 component and ~20 kDa of gp41, which are displayed on the surface of the viral membrane. Many types of gp140 trimers have been studied in efforts aimed at designing immunogens capable of eliciting protective humoral immune responses against HIV-1 infection. SOSIP gp140 trimers, developed by Moore, Sanders and colleagues, are soluble, proteolytically cleaved trimer variants stabilized by the presence of an engineered intermolecular disulfide bond between gp120 and gp41 (SOS) combined with a single residue change, I559P within gp41. We established that SOSIP trimers from both Clade-A and Clade-B strains display the same closed and open quaternary conformations as those observed for native trimeric HIV-1 Env, as assessed by cryo-electron tomography at ~20-Å resolution. As with native HIV-1 Env, the soluble trimers show similar open quaternary conformations with the binding of 17b alone, of soluble CD4 alone or of both soluble CD4 and 17b.

We have also used single-particle cryo-EM to determine the structure of the 17b–bound open conformation of soluble trimeric HIV-1 Env at a resolution of ~9 Å. These studies revealed the organization of three gp41 helices at the center of the trimer as a key structural signature of trimeric HIV-1 Env in this complex. Despite these insights into Env conformational changes, no structural information is currently available on the structure and organization of the gp120–gp41 complex in mature, proteolytically cleaved Env trimers in the native conformation, nor is there an understanding of underlying structural changes in gp41 with Env activation. Here, we report a cryo-EM structural analysis of trimeric HIV-1 Env in the closed, prefusion state. Our results provide...
new insights into the molecular architecture of trimeric Env and into the structural mechanisms involved in the entry of HIV-1 into target cells.

RESULTS
Cryo-electron microscopy of gp140–VRC03 complex
To determine the structure of the prefusion state of trimeric HIV-1 Env, we carried out cryo-EM analysis of the complex of VRC03 Fab with cleaved, soluble SOSIP trimeric HIV-1 Env, taking advantage of our earlier findings that VRC03 binding to intact HIV-1 preserves Env in the closed quaternary conformation. The bound Fab fragment increases the effective size and dimensions of the ligand-free trimer and thus improves the accuracy with which orientations can be assigned to each molecular image in the three-dimensional (3D)-reconstruction procedure. Cryo-electron micrographs recorded from vitrified suspensions of the Env–VRC03 complex show the expected dimensions for the complex (Supplementary Fig. 1a,b). Individual molecular images and class averages (Supplementary Fig. 1c) are readily recognized as representing projections of the Env–VRC03 complex previously determined by cryo-electron tomography of intact virus. We performed 3D reconstruction of the structure of the soluble Env–VRC03 Fab complex, using two different 3D-reconstruction programs (FREALIGN and RELION) independently, in order to provide greater confidence in structure determination (Supplementary Fig. 2a,b).

The final resolution for both maps, measured with the 0.143 and 0.5 Fourier-shell-coefficient cutoff criteria, is ~6 Å and ~8 Å, respectively (Supplementary Fig. 2c). We derived these density maps solely from the information present in the experimentally obtained cryo-EM images, without recourse to atomic structural models of gp120 at any stage of processing, which could otherwise result in apparent map improvement as a consequence of model bias. Features of the map that are more ordered and closer to the symmetry axis, such as the central gp41 helices, are consistent with the 6-Å resolution value, whereas features at the periphery, such as the VRC03 Fab, or less-ordered regions, such as the V1–V5 loops, are at lower resolution. There are several possible reasons for the variable resolution in different regions of the map, which could include insufficient sampling of all orientations in the particle images and limited accuracy in the assignment of orientations because of the small size of the complex, as illustrated by the point spread in the tilt-pair parameter plot (Supplementary Fig. 2d). Errors in orientation assignment of particle images are more pronounced at its periphery because those regions are the furthest from the symmetry axis, where small angular errors translate into larger displacements as compared to those of regions that are closer to the symmetry axis. It is also possible that the peripheral Fab arms have greater flexibility than do the central regions.

Molecular architecture of HIV-1 Env trimer in closed state
The density map of the VRC03–Env complex, fitted (by automated procedure) with three copies of the crystal structure of VRC03 Fab complexed to the truncated gp120 core (PDB 3SE8 (ref. 23)), reveals the overall structural organization of the complex, including the Fab arms (Fig. 1). The crystal structure includes coordinates for residues 44–123, 198–301, 325–398 and 407–492 of gp120, which are accommodated in the density map. The map also includes density for the V1/V2 loop region, the gp120 N and C termini and the gp41 base, which are absent in the crystal structure (PDB 3SE8 (ref. 23)).

A striking feature of the map is the appearance of a prominent set of three long central densities at the core of the trimer. Because all of the relevant portions of the gp120 polypeptide are accounted for within the rest of the map, these central densities are likely to arise from gp41-derived α-helical segments. At the present resolution, this density cannot be definitively assigned to a specific region of the gp41 polypeptide, but it probably includes a major contribution from the gp41 N-terminal helix region, as we suggested previously in the case of the open quaternary conformation. With the exception of this additional feature, the overall structure of the trimeric complex has all of the general features of the map obtained previously at 20-Å resolution by cryo-electron tomography.
expected on the basis of the crystal structure of the gp120–VRC03 complex (Fig. 2d). Although it is likely that there will be important differences in conformation between the structure of gp120 in the trimeric state as compared to that seen in 3D crystals that contain monomeric gp120 (ref. 25), our density map suggests that the general features of the structure of the gp120 component of the trimer, at least in the core region, are well described by the crystal structure (PDB 3SE8 (ref. 23)).

**Structural mechanism of Env activation**

A view of only the gp120 and gp41 components of the Env–VRC03 complex provides a clearer interpretation of the gp41 densities and the arrangement of the three gp120 protomers around the central gp41 stalk (Fig. 3). Most of the residues in the V1V2 loop are not included in the construct used to obtain the crystal structure of the monomeric gp120–VRC03 complex, but density for this region is evident at the top of the trimer density map. From the location of the stump of the V3 loop near the apex, it appears likely that the V3 loop is partially buried by the V1V2 loop in the closed conformation of the trimer (Fig. 3). The unassigned densities at the bottom of the map include contributions from gp41 and from the N- and C-terminal residues of gp120 (1–43 and 493–510) that are absent in the crystal structure of the gp120–VRC03 complex. The three central gp41 helices emerge from this cradle of density at the bottom and are also joined to the rest of Env near the apex at what appears as a sharp bend. The central gp41 helices are thus supported by interactions at both ends, with the rest of the gp41 ectodomain closely nestled against gp120.

Comparison of the structures of the closed, prefusion and open, activated conformations (Supplementary Fig. 6) reveals that in each case, the central densities are at the same location, despite the substantial change in location and orientation of gp120 (Fig. 4a). This remarkable conservation of the structure at the center while changes occur at the periphery suggests that the central gp41 helices in trimeric HIV-1 Env serve as an anchor around which the three gp120 protomers pivot.

**Figure 3** Molecular structure of soluble trimeric HIV-1 Env in the closed state. The density for the three VRC03 Fab moieties from the map shown in Figure 1 was computationally removed to provide a view of only the Env component of the complex, illustrating the locations of gp120, gp41 and the V1V2 and V3 loops. The density map was fit with three copies of the gp120 core (chain G of PDB 3SE8 (ref. 23)), shown in red. Residues 120–123 and 199–204 are not shown, and residues at the base of the V3 loop are shown in surface representation and colored orange. The density corresponding to the V1V2 loop is highlighted in purple, and the three central gp41 helices (derived from PDB 3HMG (ref. 23)) are shown in cyan.

**Figure 2** Detailed view of gp120 and gp41 structural elements. (a) Close-up view of the arrangement of the three gp120 protomers around the three long rods of central density (indicated by arrows), as viewed from the apex of the trimer (left) or the side (right). (b) Close-up view of the fit of the gp120 portion of PDB 3SE8 (ref. 23) coordinates into the map shown in side (left) and top (right) views. The gp41 central helices are shown in cyan in b and in the right side of a and include residues 92–122 from PDB 3HMG (ref. 23). (c) Ribbon representation of the crystal structure of the gp120 portion of PDB 3SE8 (ref. 23) coordinates, highlighting some of the major secondary-structural elements: α0 (yellow), α1 (green), α2 (orange) and α5 (gray), and a sheet formed from strands β12, β13 and β22 (magenta) in the crystal structure of gp120 (ref. 2). (d) Zoomed-in views of selected regions of the density map, illustrating quality of fit of secondary-structural elements color-coded as in c.
outwards upon ligand activation (Fig. 4b). This large conformational change, which is seen here to involve the same rearrangements as those that occur with native trimeric Env on intact virions, involves relocation of the V1V2 loop to the periphery of the trimer upon activation. The base of the V3 loop is in roughly the same position in both prefusion and activated states, but the visualization of these structures explains clearly how the outward movement of the V1V2 loop could uncloak the partially buried V3 loop in the open quaternary conformation. The preservation of similar gp41 architecture in the central region thus suggests an elegant structural mechanism for how the overall trimeric assembly remains intact despite the large quaternary structural rearrangements of gp120 that occur with transition to the open state.

**DISCUSSION**

The overall organization of trimERIC HIV-1 Env, now derived at sub-nanometer resolution in both prefusion and activated intermediate states, bears a striking resemblance to the structures of corresponding states previously observed for influenza hemagglutinin trimers. The possible similarity in spike architecture and activation mechanism between HIV, influenza and other viruses such as Ebola has long been hypothesized. In influenza hemagglutinin, three copies of HA1 are arranged around the central stalk formed by HA2 trimers in the prefusion state. Movement of HA1 protomers away from the central axis during the activation process ultimately leads to formation of the postfusion six-bundle state formed from HA2 trimers. The role of co-receptor binding in the case of the HIV-1 spike is probably analogous to the role of low pH in induction of conformational changes in the influenza spike. How these changes lead to the formation of the prehairpin intermediate state, and to the slow conversions involved in the steps leading to membrane fusion in both viruses, is still not understood in mechanistic detail. Nevertheless, our findings with HIV-1 Env showing a closed prefusion state with a central stalk made up of three α-helices and an activation process leading to an outward displacement of the three gp120 protomers provide further and strong evidence for a fundamental similarity between HIV and influenza. They extend previously noted similarities in the metastability of the prefusion state, and the architecture of the final postfusion state. Considering all of these structural and functional similarities in the fusion machinery, it is...
not surprising that the broadest human monoclonal antibodies against HIV and influenza target gp140 and HA2, the respective central anchors of these trimeric glycoproteins.  

The general similarity between the molecular architectures of the gp120–gp41 trimer in HIV-1 and the HA1–HA2 trimer in influenza suggests the likely origin of the long central helices in the prefusion spike. In both HIV and influenza, the final postfusion state of the spike involves a closely packed, six-helix-bundle arrangement (derived from HA2 or gp41) in which three central helices are surrounded by three peripheral helices. In the prefusion state and activated intermediate states of the influenza spike, the central stalk is a trimer of three long helices, each made up of a composite of three regions from HA2 (Fig. 5a). The segments at the bottom become the outer helices of the postfusion six-helix bundle, and the segments at the top become a portion of the inner helices of the six-helix bundle. The segments in the middle transform into the loops connecting the inner and outer helices (Fig. 5a). If the central helices in the HIV-1 Env prefusion state also undergo similar rearrangements to reach the final six-helix-bundle state, it seems plausible that the central helix in the closed and open quaternary states of the HIV-1 Env spike may be composed of regions that include the corresponding segments from the N-terminal and C-terminal helices in the postfusion six-helix-bundle state (Fig. 5b). This mode of packing also implies that in both the closed and open quaternary conformations, the gp41 fusion peptide is probably buried toward the base of the HIV-1 spike, as observed for hemagglutinin and surface spikes in Ebola and SARS, which represent class I fusion proteins. This model is consistent with the previous proposal that the central helix is likely to include much of the gp41 N-terminal helix region, with the bend at the top end probably representing the point where the polypeptidic is bent downwards to enable the fusion peptide to be buried.

The structural analyses we report here provide a starting point to explore the conformational changes that occur in trimeric HIV-1 Env with binding of ligands such as CD4, co-receptor and various antibodies and fusion inhibitors. As shown previously, some broadly neutralizing antibodies such as VRC01 or VRC03 can access the CD4-binding site with no change in quaternary conformation relative to the ligand-free state, whereas others such as b12 bind almost the same region of gp120 but require a partial opening to be accommodated on the surface of the trimer. The structural plasticity of the trimer is thus fundamentally related to the neutralization potency of antibodies that require quaternary structural change for efficient binding. To advance beyond the visualization of individual α-helices that we report here, and to obtain an unambiguous chain trace of the gp41 and gp120 polypeptides in the structure of trimeric Env, including all of the ordered portions of the gp120 variable loops and N-linked carbohydrate moieties on the surface, it will be necessary to obtain structures at resolutions of 4 Å or better. Recent advances in direct electron detectors for cryo-EM have enabled structure determination at 3.3-Å resolution for a highly symmetric 700-kDa complex derived from a thermophilic organism. Application of these and related technological advances to achieve similar resolutions for small, <500 kDa, dynamic protein complexes such as soluble trimers is thus an exciting and challenging frontier in structural biology. Such advances could provide a window into the detailed landscape of spike structural changes in enveloped viruses such as HIV and influenza, thus allowing characterization of intermediate states that may not always be accessible to analysis by X-ray crystallography.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**ACCESSION CODES**

The density map for the gp140–VRC03 complex and the fitted coordinates have been deposited with the Electron Microscopy Data Bank, under accession codes of EMDB-2484 and PDB 4CC8, respectively. The complete set of raw electron micrographs used to obtain the density map presented here is also available through the Electron Microscopy Data Bank and from the authors upon request.

**ACKNOWLEDGMENTS**

This work was supported by funds to S.S. and J.L.S.M. from the Center for Cancer Research at the National Cancer Institute, US National Institutes of Health (NIH), and to S.S. from the NIH Intramural AIDS Targeted Antiviral Program. We thank J. Mascola (Vaccine Research Center, NIH) for providing VRC03 antibodies; K. Kang and W. Olson (Progenics) for providing soluble KN1144 gp140 trimers; S. Fellini, S. Chacko and their colleagues for continued support with use of the Biowulf cluster for computing at NIH; D. Schauder and H. He for assistance with data collection; P. Rao and the NIH-FEI Living Lab for Structural Biology for assistance with collection of the tilt -pair images; and L. Earl for helpful discussions and comments.

**AUTHOR CONTRIBUTIONS**

A.R., A.M., M.J.B., J.L.S.M. and S.S. analyzed and interpreted data; S.S. was responsible for data collection; all authors helped compose the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Wyatt, R. & Sodroski, J. The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. *Science* **280**, 1884–1888 (1998).
2. Kwong, P.D. et al. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* **393**, 648–659 (1998).
3. Huang, C.C. et al. Structure of a V3-containing HIV-1 gp120 core. *Science* **310**, 1025–1028 (2005).
4. Zhou, T. et al. Structural definition of a conserved neutralization epitope on HIV-1 gp120. *Nature* **445**, 732–737 (2007).
5. Pancera, M. et al. Structure of HIV-1 gp120 with gp41-interactive region reveals layered envelope architecture and basis of conformational mobility. *Proc. Natl. Acad. Sci. USA* **107**, 1166–1171 (2010).
6. Zhou, T. et al. Structural basis for broad and potent neutralization of HIV-1 by antibody VRC01. *Science* **329**, 811–817 (2010).
7. Merk, A. & Subramaniam, S. HIV-1 envelope glycoprotein structure. *Curr. Opin. Struct. Biol.* **23**, 268–276 (2013).
8. Chan, D.C., Fass, D., Berger, J.M. & Kim, P.S. Core structure of gp41 from the HIV envelope glycoprotein. *Cell* **89**, 263–273 (1997).
9. Weissenhorn, W., Dessen, A., Harrison, S.C., Skelhel, J.J. & Wiley, D.C. Atomic structure of the ectodomain from HIV-1 gp41. *Nature* **387**, 426–430 (1997).
10. Bartesaghi, A. et al. Classification and 3D averaging with missing wedge correction in biological electron tomography. *J. Struct. Biol.* **162**, 436–450 (2008).
11. Frank, G.A. et al. Computational separation of conformational heterogeneity using cryo-electron tomography and 3D sub-volume averaging. *J. Struct. Biol.* **178**, 165–176 (2012).
12. Liu, J., Bartesaghi, A., Borgnia, M.J., Sapico, G. & Subramaniam, S. Molecular architecture of native HIV-1 gp120 trimers. *Nature* **455**, 109–113 (2008).
13. Meyerson, J.R. et al. Molecular structures of trimeric HIV-1 Env in complex with small antibody derivatives. *Proc. Natl. Acad. Sci. USA* **110**, 513–518 (2013).
14. Tran, E.E. et al. Structural mechanism of trimeric HIV-1 envelope glycoprotein activation. *PNAS Pathog.* 8, e1002799 (2012).
15. Harris, A. et al. Trimeric HIV-1 glycoprotein gp140 immunogens and native HIV-1 envelope glycoproteins display the same closed and open quaternary molecular architectures. *Proc. Natl. Acad. Sci. USA* **108**, 11440–11445 (2011).
16. Sanders, R.W. et al. Stabilization of the soluble, cleaved, trimeric form of the envelope glycoprotein complex of human immunodeficiency virus type 1. *J. Virol.* **76**, 8875–8889 (2002).
17. Moscoso, C.G. et al. Quaternary structures of HIV Env immunogens exhibit conformational vicissitudes and interface diminution elicited by lipid binding. *Proc. Natl. Acad. Sci. USA* **108**, 6091–6096 (2011).
18. Wu, S. et al. Single-particle cryoelectron microscopy analysis reveals the HIV-1 spike as a tripod structure. *Proc. Natl. Acad. Sci. USA* **107**, 18844–18849 (2010).
19. Wu, S. et al. Fab-based single particle cryoEM studies of small proteins. *Structure* **20**, 582–592 (2012).
20. Grigorieff, N. FREALIGN: high-resolution refinement of single particle structures. *J. Struct. Biol.* **157**, 117–125 (2007).
21. Scheres, S.H. RELION: implementation of a Bayesian approach to cryo-EM structure determination. *J. Struct. Biol.* **180**, 519–530 (2012).
22. Pettersen, E.F. et al. UCSF Chimera: a visualization system for exploratory research.
and analysis. J. Comput. Chem. 25, 1605–1612 (2004).
23. Wu, X. et al. Focused evolution of HIV-1 neutralizing antibodies revealed by structures and deep sequencing. Science 333, 1593–1602 (2011).
24. Mao, Y. et al. Molecular architecture of the uncleaved HIV-1 envelope glycoprotein trimer. Proc. Natl. Acad. Sci. USA 110, 12438–12443 (2013).
25. Kwon, Y.D. et al. Unliganded HIV-1 gp120 core structures assume the CD4-bound conformation with regulation by quaternary interactions and variable loops. Proc. Natl. Acad. Sci. USA 109, 5663–5668 (2012).
26. Fontana, J., Cardone, G., Heymann, J.B., Winkler, D.C. & Steven, A.C. Structural changes in Influenza virus at low pH characterized by cryo-electron tomography. J. Virol. 86, 2919–2929 (2012).
27. Xu, R. & Wilson, I.A. Structural characterization of an early fusion intermediate of influenza virus hemagglutinin. J. Virol. 85, 5172–5182 (2011).
28. Harrison, S.C. Viral membrane fusion. Nat. Struct. Mol. Biol. 15, 690–698 (2008).
29. Weissenhorn, W., Carli, A., Lee, K.H., Skehel, J.J. & Wiley, D.C. Crystal structure of the Ebola virus membrane fusion subunit, GP2, from the envelope glycoprotein ectodomain. Mol. Cell 2, 605–616 (1998).
30. Wilson, I.A., Skehel, J.J. & Wiley, D.C. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. Nature 289, 366–373 (1981).
31. Bullough, P.A., Hughson, F.M., Skehel, J.J. & Wiley, D.C. Structure of influenza haemagglutinin at the pH of membrane fusion. Nature 371, 37–43 (1994).
32. Ivanovic, T., Choi, J.L., Whelan, S.P., van Oijen, A.M. & Harrison, S.C. Influenza-virus membrane fusion by cooperative fold-back of stochastically induced hemagglutinin intermediates. eLife 2, e00333 (2013).
33. Eckert, D.M. & Kim, P.S. Mechanisms of viral membrane fusion and its inhibition. Annu. Rev. Biochem. 70, 777–810 (2001).
34. Huang, J. et al. Broad and potent neutralization of HIV-1 by a gp41-specific human antibody. Nature 491, 406–412 (2012).
35. Corti, D. & Lanzavecchia, A. Broadly neutralizing antiviral antibodies. Annu. Rev. Immunol. 31, 705–742 (2013).
36. McLellan, J.S. et al. Structure of RSV fusion glycoprotein trimer bound to a prefusion-specific neutralizing antibody. Science 340, 1113–1117 (2013).
37. Baquero, E. et al. Intermediate conformations during viral fusion glycoprotein structural transition. Curr. Opin. Virol. 3, 143–150 (2013).
38. Li, X. et al. Electron counting and beam-induced motion correction enable near-atomic-resolution single-particle cryo-EM. Nat. Methods 10, 584–590 (2013).
39. Weis, W.I., Brunger, A.T., Skehel, J.J. & Wiley, D.C. Refinement of the influenza virus hemagglutinin by simulated annealing. J. Mol. Biol. 212, 737–761 (1990).
ONLINE METHODS

Proteins and preparation of specimens for microscopy. Purified samples of soluble Clade-A strain KNH1144 SOSIP, cleaved HIV-1 Env gp140 trimers containing the entire Env ectodomain including the membrane-proximal external region (MPER, residues 661–681) were kindly provided by K. Kang and W. Olson (Progenics Inc.) and were the same as those previously described and used for the structural analysis of soluble Env structure at 20 Å resolution. Purified VRC03 IgG was kindly provided by J. Mascella (Vaccine Research Center, NIAID, NIH). VRC03 Fab fragments were prepared by papain digestion, concentrated to 3 mg/ml, and incubated on ice for 30–60 min with soluble Env trimers (650 µg/ml) with 5x estimated molar excess of Fab. 2.5 µl of the mixture was deposited on 400-mesh C-flat grids from Protiochips (Raleigh, NC) with 2-µm-wide holes spaced by 4 µm, and vitrified specimens for cryo-EM were prepared with a Mark IV Vitrobot from FEI Company (Hillsboro, OR).

Imaging conditions. Plunge-frozen specimens of the Env–VRC03 Fab complex were imaged with a Titan Krios electron microscope aligned for parallel illumination at an operating voltage of 80 kV. Electron micrographs were collected on a Gatan 4,096 x 4,096 CCD optimized to maximize detective quantum efficiency (DQE) at lower voltages. The use of the lower voltage comes at the expense of sacrificing the higher microscope performance at 300 kV, but the better DQE performance provides increased image contrast, and this in turn improves image alignment accuracy. Data collection used EPU automated data-acquisition software, a total electron dose of 10 electrons/Å²/image and a nominal microscope magnification of 75,000x corresponding to a pixel size at the specimen plane of 1.08 Å. A total of 4,713 micrographs were collected, with roughly equal numbers of images recorded at defocus values of –1.5, –1.8, –2, –2.35 and –2.5 µm. From these, the subset of micrographs displaying the highest-resolution Thon- ring profiles, the least astigmatism and clearly visible, well-separated particles were then selected for further analysis.

Image processing. Hot-pixel removal to correct defects in EM images was done with IMOD's ccderaser with options ‘-scan 10’-xyscan 128’-edge 64’-radius 2.1’. CTFFIND3 (ref. 41) was used to estimate the defocus of each micrograph with an FFT box size of 512 pixels with microscope parameters for Cs and WGH of 2.7 and 0.1, respectively. The resolution range used for defocus estimation was set from 50 Å to 7 Å, with a defocus step size of 250 Å and astigmatism step size of 2.50 Å.

Particles were picked manually from 8x-binned versions of the original micrographs with EMAN's boxer and then subsequently extracted with the batchboxer command with a box size of 256 pixels, ignoring particles that extended outside the micrographs. Particle stacks were normalized with EMAN’s proc2d with option ‘edgenormalize’. All entities that had densities consistent with sizes of ~200 Å in diameter were initially selected. Characteristic top views showing the three-fold symmetry were easily recognizable and more abundant, but side and edge-on views orthogonal to the three-fold symmetry axis were also selected. That were too close to each other were not included. 114,713 particles from 3,755 micrographs were selected initially, giving an average of ~30 particles per image.

In order to obtain a clean subset of particles, 2D classification of the 114,713 particles into 500 classes was done in IMAGIC using the MSA command with the MODULATION distance for 25 iterations with 25 eigenimages. This analysis was done without precentering or aligning the particles in order to rule out any type of reference bias. Classification was done with the HAC option using all 25 eigenimages, and a 0.15 fraction of worst class members were removed from the analysis. 48 classes were visually identified as being of poor quality or heterogeneous and were deselected, thus giving a reduced data set of 88,125 particles that were subsequently used for 3D refinement. The particle selection and classification were independently carried out numerous times to verify that consistent class averages were obtained each time. The class averages (Supplementary Fig. 1c) invariably showed agreement with the projection images expected from the tomographically reconstructed density map of the native Env–VRC03 complex at 20 Å resolution (Supplementary Fig. 3a) that was used as a starting model for 3D refinement.

Single-particle refinement with the tomographic map as an initial model was done with FREALIGN v8.10 (ref. 20) and RELION v1.2 (ref. 21) imposing three-fold (C3) symmetry. FREALIGN refinement was carried out with frequency components between 100 Å and 8 Å as follows: four initial rounds of orientation assignment with randomized global search (MODE 4) were done with parameters DANG = 200 and ITMAX = 50, and this was followed by four additional rounds of local refinement (MODE 1). At each iteration round, the top 75% of particles according to phase residual were included in the reconstruction, and a value of PBC = 100 was used in all iterations. RELION refinement with the ‘3D auto- refine’ option was carried out for 19 iterations. Refinement parameters were as follows: CTFs until first peak were ignored, and an initial low-pass filter of 20 Å was used. The particle diameter was set to 220 Å, and no reference mask was used. The initial angular-sampling interval was set to the default value of 7.5 degrees, and the offset search range and offset search step were also set to default values of 5 and 1 pixels, respectively. Local searches from autosampling were set to the default value of 1.6 degrees as well.

Maps obtained with either FREALIGN or RELION were verified to display the same salient structural features, and they displayed similar resolution values in Fourier shell correlation (FSC) plots (Supplementary Fig. 2a–c). FSC resolution plots were obtained with EMAN’s proc3d command with the unfiltered half maps with soft spherical masks applied to eliminate the influence of the background in the resolution estimates. The curves show the ‘gold-standard’ curve obtained with RELION and the curve obtained from the correlation of two halves of the data set obtained with FREALIGN, and indicate resolution values of ~6 Å at 0.143 FSC and ~8 Å at 0.5 FSC cutoff values. For the purpose of visualization, maps were corrected by a B factor of ~600 Å2 and low-pass–filtered to the 0.143 FSC–cutoff resolution of 6 Å. The final map was also validated with the tilt-pair parameter plot (Supplementary Fig. 2d). 383 particles were selected manually from 19 sets of tilt-pair images with e2RCTboxer.py in the EMAN2 suite.

With the final density map as the reference 3D model, particle orientations were assigned with FREALIGN and plotted with TILTMULTIDIFF. The spread of angular orientations for most of the tilt pairs is within ~12.5°, values consistent with expectations of a density map at the resolution obtained for a complex with a polypeptide mass of ~400 kDa. Fits of coordinates to the density map and rendering of maps and coordinates were carried out with UCSF Chimera.

Figure 1c

Maps obtained with either FREALIGN or RELION were verified to display the same salient structural features, and they displayed similar resolution values in Fourier shell correlation (FSC) plots (Supplementary Fig. 2a–c). FSC resolution plots were obtained with EMAN’s proc3d command with the unfiltered half maps with soft spherical masks applied to eliminate the influence of the background in the resolution estimates. The curves show the ‘gold-standard’ curve obtained with RELION and the curve obtained from the correlation of two halves of the data set obtained with FREALIGN, and indicate resolution values of ~6 Å at 0.143 FSC and ~8 Å at 0.5 FSC cutoff values. For the purpose of visualization, maps were corrected by a B factor of ~600 Å2 and low-pass–filtered to the 0.143 FSC–cutoff resolution of 6 Å. The final map was also validated with the tilt-pair parameter plot (Supplementary Fig. 2d). 383 particles were selected manually from 19 sets of tilt-pair images with e2RCTboxer.py in the EMAN2 suite.

With the final density map as the reference 3D model, particle orientations were assigned with FREALIGN and plotted with TILTMULTIDIFF. The spread of angular orientations for most of the tilt pairs is within ~12.5°, values consistent with expectations of a density map at the resolution obtained for a complex with a polypeptide mass of ~400 kDa. Fits of coordinates to the density map and rendering of maps and coordinates were carried out with UCSF Chimera.

40. Kremer, J.R., Mastronarde, D.N. & McIntosh, J.R. Computer visualization of three-dimensional image data using IMOD. J. Struct. Biol. 116, 71–76 (1996).
41. Mindell, J.A. & Grigorieff, N. Accurate determination of local defocus and specimen tilt in electron microscopy. J. Struct. Biol. 142, 334–347 (2003).
42. Ludtke, S.J., Baldwin, P.R. & Chiu, W. EMAN: semiautomated software for high-resolution single-particle reconstructions. J. Struct. Biol. 128, 82–97 (1999).
43. van Heel, M. et al. in International Tables for Crystallography Volume F: Crystallography of Biological Macromolecules 2nd edn (eds Arnold, E., Himmel, D.M. & Rossmann, M.G.) 624–628 (Wiley, 2012).
44. Henderson, R. et al. Tilt-pair analysis of images from a range of different specimens in single-particle electron cryomicroscopy. J. Mol. Biol. 413, 1028–1046 (2011).
45. Rosenthal, P.B. & Henderson, R. Optimal determination of particle orientation, absolute hand, and contrast loss in single-particle electron cryomicroscopy. J. Mol. Biol. 333, 721–745 (2003).
46. Tang, G. et al. EMAN2: an extensible image processing suite for electron microscopy. J. Struct. Biol. 157, 38–46 (2007).
Corrigendum: Prefusion structure of trimeric HIV-1 envelope glycoprotein determined by cryo-electron microscopy

Alberto Bartesaghi, Alan Merk, Mario J Borgnia, Jacqueline L S Milne & Sriram Subramaniam
Nat. Struct. Mol. Biol.; doi:10.1038/nsmb.2711; corrected online 10 November 2013

In the version of this article initially published online, the NIH-FEI Living Lab for Structural Biology was not mentioned in the Acknowledgments section. The error has been corrected for the print, PDF and HTML versions of this article.