**Structural Biology**

**Structural basis for membrane anchoring of HIV-1 envelope spike**

Jyoti Dev, Donghyun Park, Qingshan Fu, Jia Chen, Heather Jiwon Ha, Fadi Ghantous, Tobias Herrmann, Weiting Chang, Zhijun Liu, Gary Frey, Michael S. Seaman, Bing Chen, James J. Chou

HIV-1 envelope spike (Env) is a type I membrane protein that mediates viral entry. We used nuclear magnetic resonance to determine an atomic structure of the transmembrane (TM) domain of HIV-1 Env reconstituted in bicelles that mimic a lipid bilayer. The TM forms a well-ordered trimer that protects a conserved membrane-embedded arginine. An aminoterminal coiled-coil and a carboxyl-terminal hydrophilic core stabilize the trimer. Individual mutations of conserved residues did not disrupt the TM trimer and minimally affected membrane fusion and infectivity. Major changes in the hydrophilic core, however, altered the antibody sensitivity of Env. These results show how a TM domain anchors, stabilizes, and promotes membrane fusion and infectivity. Major changes in the hydrophilic core, however, altered the antibody sensitivity of Env. The cryo-EM (electron microscopy) structure of a detergent-solubilized trimer (theoretical molecular mass 14.1 kDa) (fig. 1F) suggests clustering of TM helices (2–9). Hence, the TM domain anchors, stabilizes, and promotes membrane fusion and infectivity. Major changes in the hydrophilic core, however, altered the antibody sensitivity of Env. The cryo-EM (electron microscopy) structure of a detergent-solubilized trimer (theoretical molecular mass 14.1 kDa) (fig. 1F) suggests clustering of TM helices (2–9). Hence, the TM domain anchors, stabilizes, and promotes membrane fusion and infectivity.

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17. Materials and methods are available as supplementary materials on Science Online.

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**Supplementary materials**

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Materials and Methods

Figs. S1 to S3

Tables S1 to S6

References (21–70)

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gp41<sub>HIV1D(677-716)</sub> is a tightly assembled trimer ~54 Å long, with the conserved arginine (R696) near its midpoint (Fig. 1A). It shows a packing arrangement not seen in any other known TM helix dimers or trimers: Its N- and C-terminal halves have different modes of assembly, with an intervening kink. The N-terminal region is a conventional three-chain coiled-coil formed by residues 686 to 696 (Fig. 1B), including the GxxxG motif. The C-terminal half does not show classic “knobs-into-holes” interactions, but instead is held together by a network of polar contacts, mainly involving R707 and Q710, at the trimer interface of the kinked helical segments (residues 704 to 712) (Fig. 1C). We call this interface the “hydrophilic core.”

R696, near the middle of each TM helix (Fig. 1D), produces three unbalanced charges at the membrane R696 and its surrounding hydrophobic residues, as well as the contremporary backbone oxygen of L692 and the water molecule. The polarizability of the hydrophobic pocket that surrounds the guanidinium group, presumably indicative of the presence of an adjacent structured water. Thus, the guanidinium group, presumably charged at pH 6.7 under the NMR conditions, is partially neutralized by hydrogen bonding with the electronegative backbone oxygen of L692 and the water molecule. The polarizability of the hydrophobic pocket that surrounds the guanidinium group, may also lower its pK<sub>a</sub> (acid dissociation constant) from its value in aqueous solution. Although well accommodated in the TMD trimer, the intramembrane R696 may modulate the stability of the helical trimer if the helices dissociate at any stage in assembly or fusion. The <sup>1</sup>H-<sup>15</sup>N correlation spectrum of the gp41<sub>HIV1D(677-716)</sub> trimer showed inhomogeneous peak linewidth, with the N-terminal half near R696 having the most severe peak broadening, consistent with conformational fluctuation (fig. S7).

To confirm membrane partition of the TMD trimer, we used a paramagnetic probe, Gd(DOTA) (24), to measure solvent exposure of the four arginine residues in the gp41<sub>HIV1D(677-716)</sub> trimer. These arginines are distributed at different positions along the TM helices and thus serve as four depth markers. We measured intensity decrease of the arginine H-N<sub>c</sub> correlation peaks at increasing concentrations of Gd(DOTA). The most solvent-exposed R707 showed the highest sensitivity to Gd(DOTA), whereas the most buried R696 was the least sensitive (Fig. 2A). R683 and R709 are near opposite lipid headgroup regions in the structure. R709 showed a greater resonance broadening than did R683, indicating that the latter is more deeply buried. We placed the gp41<sub>HIV1D(677-716)</sub> trimer in the lipid bilayer so that the four arginine positions were consistent with their respective sensitivity to Gd(DOTA) (Fig. 2B). This placement, which is consistent with the surface distribution of hydrophobic, polar, and charged residues (Fig. 2C), places R696 in a fully hydrophobic environment, slightly closer to the cytoplasmic side of the membrane. The MPER segment is in the headgroup region of the outer leaflet. The C-terminal segment, previously assigned to the CT, is at the headgroup-water interface of the inner leaflet.

To assess the contribution of specific residues to TMD stability, we generated 12 gp41<sub>HIV1D(677-716)</sub> mutants with single or double mutations, mainly...
was resistant to most neutralizing antibodies, similar cell-surface levels (figs. S9 and S10). At a of cleavage between gp120 and gp41, as well as ly transfected in 293T cells, all mutants expressed these elements (table S2). We also produced mukink, the hydrophilic core, or combinations of coil region, R696 and its protecting residues, the Activity to either antibody; mutants I686A, Y712A, and G690L/P714H exhibited detectable differences in infectivity for most of them (table S2 and figs. S13 and S14). R696A had almost full wild-type infectivity, whereas 704–713 and G690L/704–713 had substantially less—just the opposite of their effects on cell-cell fusion (table S2 and fig. S14).

To determine whether mutations in the TMD can influence the antigenic structure of the Env ectodomain and whether they might affect its antigenic structure, we used the cell-cell fusion assay to test inhibition of each Env mutant by a trimer-specific bnAb PGI6 and by a nonneutralizing V3 antibody 3791 (table S2). Most mutants were essentially identical to the wild type in their sensitivity to either antibody; mutants I686A, Y712A, P714N, G690L/Y712A, G690L/P714N, G690L/P714K, and G690L/P714H exhibited detectable differences (fig. S15). We observed the most pronounced differences for mutations 704–713 and G690L/704–713, for which PGI6 and 3791 switched phenotypes—the former became inactive and the latter inhibitory (fig. S15). All mutations that affect antibody inhibition are located in either the coiled-coil or the hydrophilic core. We infer that changes in TMD stability influence the antigenic structure of the ectodomain of the functional Env. When tested in a pseudovirus-based neutralization assay with bnAbs P9G (trimer specific), 3BNC117 (CD4 binding site), 10-1074 (glycan- and V3-dependent), and 3971 (table S3), most mutants, except for 704–713 and G690L/704–713, were unchanged in their sensitivity to PG9, 3BNC117, 10-1074 and 3791, but most became more sensitive to 10E8 (table S4). This result suggests that the changes produced by all the mutations tested, except for 704–713 and G690L/704–713, are limited to local structure. In contrast, the mutants 704–713 and G690L/704–713 became resistant to PG9 and sensitive to 3791—reflecting their properties in the cell-cell fusion assay. Mutant 704–713 was analyzed with additional antibodies. For cell-cell fusion, wild-type Env is sensitive to trimer-specific bnAbs PG9, PG16, and PGT145 and resistant to nonneutralizing antibodies b6 (CD4 binding site), 3791, and 17b (CD4-induced) (table S3, Fig. 3A, and fig. S16). The antibody inhibition pattern is reversed, however, for the fully functional mutant 704–713, indicating that the hydrophilic core of the TMD plays an important role in stabilizing and modulating the antigenic structure of the Env spike. Similar phenotypes were also observed with a 704–713 mutant derived from a clade C strain C972A012 (fig. S17). The pseudovirus neutralization assay gave similar, but less pronounced, results for the mutant 704–713 (Fig. 3B and table S4).

The most important finding from this study relevant to vaccine development is how the TMD modulates the antigenic surfaces of the Env spike. We reported previously that truncation of the CT domain of HIV-1 Env reshapes the antigenic surfaces of its ectodomain (13). We now show that mutations destabilizing the hydrophilic core of the TMD trimer resemble the CT deletion in altering the sensitivity of the functional Env to both nonneutralizing and trimmer-specific neutralizing antibodies. In particular, the trimer-specific bnAbs, which neutralize by stabilizing the native
Fig. 3. Effect of mutations in the TMD of HIV-1 Env on its antibody sensitivity. (A) Antibody inhibition of cell-cell fusion mediated by the wild-type 92UG0378 Env (left) and the TMD mutant 704–713 [right; residues 704 to 713 (VINVQRQGYS) were mutated to SSAASAGSA] was analyzed with both nonneutralizing antibodies—including b6 (CD4 binding site; blue), 3791 (V3; cyan), and 17b (CD4-induced; purple)—and trimer-specific bnAbs, including PG9 (orange), PG16 (red), and PGT145 (magenta). The CD4 binding site bnAb VRC01 (green) was a control antibody. The experiment was carried out in triplicate and repeated at least twice with similar results. Error bars indicate the SD calculated by the Excel STDEV function. (B) Antibody neutralization of pseudoviruses containing either the 92UG0378 Env (left) or the TMD mutant 704–713 (right) was determined with antibodies b6, 3791, 17b, PG9, PG16, and PGT145, shown in the same color scheme as in (A). The CD4 binding site bnAb 3BNC117 was a control antibody (green). The experiment was performed in duplicate.

conformation of Env (26, 27), do not recognize the Env spike when its TMD has been destabilized. We suggest that the TMD mediates conformational coupling between the ectodomain and the CT and that the trimeric structure seen by NMR represents the conformation of the TMD adopted by a native Env spike in a membrane.

The placement of sp441 (N2067T776) in a lipid bilayer reveals clear boundaries of the TM segment and settles a contentious issue (20, 28). Part of the 10E8 epitope (residues 677 to 683) is embedded in the headgroup layer of the outer leaflet, consistent with lack of accessibility of this epitope on the native Env (33, 29). The hydrophilic core, which was thought to be part of the CT, is similarly protected by the headgroup layer of the inner leaflet. This hydrophilic region contains a tyrosine-based sorting signal (72GYPSP725), which may participate in Env internalization by endocytosis (15, 16, 30). Our structure indicates that Y712 and P714 in this motif on one TM protomer interact with L704 and V708 of the adjacent protomer, respectively, thereby also contributing to trimer stability.

The TMD is required not only for membrane anchoring and fusion, but also for stability of the entire Env spike. This observation can explain why most soluble Env preparations with the TMD deleted, except for those of a few selected strains (31, 32), are unstable and conformationally heterogeneous, unless they have specific, stabilizing modifications (26, 33, 34). To design immunogens that mimic optimally native viral spikes, one must not ignore structural constraints imposed by the TMD on the ectodomain. The high-resolution structure of the HIV-1 Env TMD trimer presented here can be a guide for engineering more effective immunogens.

REFERENCES AND NOTES
19. To produce the protein, we expressed the His-tagged TrpLE-gp41(16106–1776) fusion protein in Escherichia coli as inclusion bodies, purified the solubilized protein by Ni-affinity chromatography, removed the TrpLE tag with cyanogen bromide, and separated the product by reverse-phase high-performance liquid chromatography. Bicelles were made of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC; lipid) and 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC; detergent) at a ratio (v/v) of 1:0.8. The bicelles were separated by reverse-phase high-performance liquid chromatography. Bicelles were made of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC; lipid) and 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC; detergent) at a ratio (v/v) of 1:0.8. In this report, we use gp41(16106–1776) and TMD interchangeably for convenience.
23. For structure determination, we proceeded with the crystal D construct because its expression level was the highest. The approach involves determination of local structures of the monomers and assembly of the trimer with intermonomer distance restraints derived from NOEs between structurally equivalent but isotopically differently labeled subunits. We could identify eight intermonomer NOEs using the isotopically mixed labeled sample to calculate a unique assembly solution, which was further validated and refined with other conventional NOE data.
24. Gd(DOTA) is a water-soluble and membrane-inaccessible molecule, so that the paramagnetic relaxation enhancement (PRE) it generates decreases with distance from the bilayer surface.

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SUPPLEMENTARY MATERIALS
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Editor's Summary

**Env's transmembrane domain revealed**

HIV-1's envelope protein (Env) spans the viral membrane and grants the virus entry into host cells. Env is also the sole protein of HIV-1 that is targeted by antibodies, making it a key target for vaccine design. Dev et al. used nuclear magnetic resonance to determine an atomic-level structure of the membrane-spanning region of Env in a lipid bicelle. Env's transmembrane domain forms a well-ordered trimer, which includes a stabilizing C-terminal hydrophilic core. Disrupting this core alters the sensitivity of Env to broadly neutralizing antibodies, suggesting the potential importance of this region to vaccine design.

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