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The HIV-1 envelope glycoprotein structure: nailing down a moving target

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Summary

Structure determination of the HIV-1 envelope glycoprotein (Env) presented a number of challenges, but several high-resolution structures have now become available. In 2013, cryo-EM and x-ray structures of soluble, cleaved SOSIP Env trimers from the clade A BG505 strain provided the first glimpses into the Env trimer fold as well as more the variable regions. A recent cryo-EM structure of a native full-length trimer without any stabilizing mutations had the same core structure, but revealed new insights and features. A more comprehensive and higher resolution understanding of the glycan shield has also emerged, enabling a more complete representation of the Env glycoprotein structure. Complexes of Env trimers with broadly neutralizing antibodies have surprisingly illustrated that most of the Env surface can be targeted in natural infection and that the neutralizing epitopes are almost all composed of both peptide and glycan components. These structures have also provided further evidence of the inherent plasticity of Env and how antibodies can exploit this flexibility by perturbing or even stabilizing the trimer to facilitate neutralization. These breakthroughs have stimulated further design and stabilization of Env trimers as well as other platforms to generate trimers that now span multiple subtypes. These Env trimers when used as immunogens, have led to the first vaccine-induced neutralizing antibodies for structural and functional analyses.

KEYWORDS
cryo-electron microscopy, epitope mapping, glycan shield, HIV envelope structure, structure-based vaccine design, x-ray crystallography

1 | INTRODUCTION

Elucidation of the three-dimensional structure of the HIV-1 Env trimer was thwarted for many years due the extreme challenges in isolating or designing an Env protein that was stable enough and could be produced in sufficient quantity and purity for high-resolution studies. Viral glycoproteins in general have been difficult to study with the exception of influenza hemagglutinin and neuraminidase, which were isolated by cleavage from the virus surface over 40 years ago using proteases,[1–3] thereby enabling low-resolution views first by electron microscopy[4,5] and then high-resolution structures by X-ray crystallography.[6,7] However, for other viral glycoproteins, development of

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recombinant expression systems, as well as stabilized soluble versions of the glycoproteins, were essential for successful structure determination. Even for influenza hemagglutinin, a robust recombinant expression system that could provide sufficient quantities for structural studies was not developed until 2004.[8]

Despite the best efforts from the community worldwide, it was not until the development of stabilized Env trimers by John Moore, James Binley and Rogier Sanders that a promising solution was found for HIV-1 Env. Engineering of a disulfide (SOS) between gp120 and gp41 to covalently hold the subunits together[9, 10] and an isoleucine to proline mutation in the HR1 region of gp41 to aid in trimer formation led to a stabilized trimer called SOSIP[11] that finally enabled a soluble, cleaved Env trimer to be assembled and expressed (reviewed in this issue by Sanders and Moore).[12] But it took another 11 years to find a candidate Env with optimal solubility, stability, as well as sufficient expression levels, for high-resolution structures to be undertaken by cryo-electron microscopy (cryo-EM)[13] and by X-ray crystallography.[14]

Other viral glycoproteins have proved equally challenging and production of recombinant and engineered proteins were also essential to determine their three-dimensional structures. Some examples include the F glycoprotein parainfluenza virus 5,[15] glycoprotein G from vesicular stomatitis virus,[16] the fusion protein from respiratory syncytial virus,[17, 18] the Ebola virus glycoprotein,[19–21] an E2 core protein from hepatitis C virus,[22,23] the human coronavirus spike glycoprotein,[24] the prefusion glycoprotein of LCMV,[25] full-length herpes simplex virus 1 glycoprotein B,[26] the Phleboviral envelope glycoprotein,[27] the Puumala virus Gn glycoprotein,[28] and hemagglutinin-esterases from rat coronavirus and mouse hepatitis virus.[29] Many of these are very recent structures that illustrate the significant advances that have been made in structure determination of viral glycoproteins.

2 | HIV-1 GLYCOPROTEIN STRUCTURES—THE FIRST GENERATION

The first structures of any Env components came via crystal structures of the postfusion conformation of gp41 HR1 and HR2 helical domains from Peter Kim, Don Wiley, and Min Lu in 1997.[30–32] The following year, Peter Kwong, Rich Wyatt, Joe Sodroski, and Wayne Hendrickson, after many years of innovative work, determined the crystal structure of the gp120 core domain of HIV-1 in 1998 in complex with soluble CD4 (sCD4) and a CD4i neutralizing antibody, 17b.[33] For many years, the gp120 core became the gold standard for analyzing gp120 and its interaction with antibodies and other ligands, and gradually its completeness was increased through adding variable loops, such as V3,[34] and regions of the N- and C-termini of gp120 that interact with gp41.[35] Eventually unliganded structures of gp120 were obtained that looked remarkably similar to the receptor-bound forms.[36]

In the interim, description and analysis of Env trimers remained in the domain of electron microscopy, particularly cryo-electron tomography (cryo-ET).[37–39] Low resolution snapshots of the Env trimer of the surface of both SIV and HIV[37] generated controversy over the exact nature of the trimer configuration and whether the base resembled a tripod[38] or a trimeric helical bundle,[39] a question that still remains unanswered. It became clear that binding of CD4 induced changes in the trimer and was much more open at the trimer apex after CD4 and Fab 17b (a CD4i antibody) were bound to the CD4 binding site (CD4bs).[40] The flexible nature of the trimers thus became apparent from this early cryo-ET work and further studies by cryo-EM revealed multiple conformations of the Env trimer that were related to the conformational rearrangements that the trimer must undergo to attain its fusion-active form after receptor binding[41] as well as a structure of the prefusion form.[42] Importantly, some antibodies were shown to stabilize the prefusion, closed form of the trimer.[41]

For soluble forms of the trimer, structure determination proceeded slowly despite the advent of the SOSIP trimer. In our own lab, we tried to crystallize the original clade B JR-FL SOSIP trimer in collaboration with John Moore and Rogier Sanders starting in 2002. It was not until much later that we found out why that did not work (see below). A change of the Env sequence to a clade A KNH1144 strain in complex with Fab 17b led to Rob Pejchal in the Wilson lab obtaining beautiful crystals with excellent morphology that indeed diffracted x-rays, but only to 17 Å resolution. It became clear that aggregation of the trimer was a problem with this construct and, after considerable experimentation, truncation of the hydrophobic MPER at residue 664 instead of at residue 681 in the original construct substantially improved the biophysical properties and eliminated the micelle around the gp41 base as clearly visualized by negative-stain EM.[43,44] The resulting crystals from this material now diffracted to 7.7 Å in complex with a different Fab, PGT123, which interacts with the high mannose patch centered around glycan N332. To further improve Env properties, SOSIP constructs were made for many different strains. Electron microscopy led to a critical advance here as, of all the constructs screened, a clade A BG505 SOSIP trimer looked the best in negative-stain EM in terms of trimers that were compact, properly folded, and emulated trimers on the surface of the virus.[45] Thus, this BG505 trimer was selected for further immunological and structural studies and was found to be very stable with a melting temperature (Tm) of 68.1°C, bound all known broadly neutralizing antibodies (except MPER Abs as MPER was not present in the construct), and was highly homogeneous and folded into a native-like structure as evaluated quantitatively by negative–stain EM.[46] The importance of cleavage between gp120 and gp41 for attaining a native conformation was also demonstrated to be critical.[47]

This soluble, cleaved BG505 SOSIP trimer then became the template for achieving the long-awaited Env trimer structure. Broadly neutralizing antibodies were also to play a crucial role in the structure determination, both by X-ray crystallography and by cryo-electron microscopy. Until 2008, only a handful of bnAbs were available to facilitate crystallization of the highly glycosylated trimer, but significant advances in methods for isolating human monoclonal antibodies against HIV [reviewed (48)] either by direct neutralization screening of single B cell cultures[49, 50] or from antigen-specific B cell sorting[51, 52] provided a rich and plentiful assortment for structural and functional studies. Indeed, the recombinant BG505 SOSIP trimer itself was later used to select for some of the most potent antibodies to date against the Env trimer.[53, 54] As a result, many antibodies became available to aid in crystallization of the Env trimer. However, heterogeneity of the glycosylation on Env was still a problem until partial deglycosylation of the antibody-SOSIP trimer complexes using EndoH was able to create more homogeneous specimens,[14] as we used previously for Fab PGT135/gp120 complexes.[55] Diffraction-quality crystals were then obtained by Jean-Philippe Julien in the Wilson lab after making a myriad of complexes of the BG505 SOSIP Env with 50 different Fabs, deglycosylating, then setting up over 100 000 crystallization trials, and screening >1000 crystals to find a crystal that diffracted sufficiently well to determine the Env structure. Thus, after more than 10 years from the first attempts to crystallize the JR-FL SOSIP trimer, suitable crystals were obtained for the BG505 SOSIP trimer.

But that was not the end of the story- far from it. The Ward lab had been using EM to select and characterize suitable Env trimers for structural study.[43, 45] However, while these EM studies were initially intended to aid the Env structural work by x-ray, major advances in cryo-EM suggested the possibility of now determining structures from single particles at sub-nanometer resolution.[56] To facilitate the detection and alignment of the Env particles, CD4bs antibody PGV04 was complexed with the soluble BG505 SOSIP trimer and, after considerable effort to overcome sample aggregation and preferred orientations, a cryo-EM structure was determined by Dmitry Lyumkis in the Ward lab at approximately 5.8 Å resolution. Furthermore, the low resolution EM envelope of BG505 bound to PGT122 was crucial for the molecular replacement solution of the X-ray structure.[14] Thus, EM not only caught up, but in a sense surpassed X-ray to achieve the structure of this challenging glycoprotein as the first views at high resolution came from cryo-EM. Also, as no crystallization was required, all of the native glycans could be left on the Env protein enabling visualization of a more native-like and complete glycan shield in addition to the protein components. The resulting structures of BG505 SOSIP by cryo-EM in complex with PGV04 (CD4bs)[13] and x-ray in complex with PGT122 (high mannose N332 glycan patch)[14] at 5.8 Å and 4.7 Å resolution, respectively, provided the first atomic-level views of a soluble Env trimer. These structures disclosed the intimate association of the gp120 and gp41 components, the prefusion helical structure of gp41 with its central triple coiled-coil HR1 helices and the close interaction of the hypervariable V2 and V3 loops around the Env trimer apex (Figures 1 and 2). Furthermore, broadly neutralizing epitopes for the CD4 binding site and the high mannose N332 patch were visualized in the context of the trimer, illustrating why it was so difficult to raise bnAbs from individual components of the trimer, such as gp120, because antibodies can approach gp120 in ways that are not permitted in a trimer context (i.e. non-neutralizing antibodies are usually generated using gp120 or malformed trimers as an immunogen[57]). Both EM and X-ray structures highly complemented each other and laid the foundation for understanding the intrinsic beauty and sheer complexity of the Env trimer structure and then for how to design other versions of soluble trimers from different strains and subtypes.[58]

3 | HIV-1 GLYCOPROTEIN STRUCTURES—THE NEXT GENERATION

The next major step was to improve the resolution of the Env trimer structure. Peter Kwong and colleagues utilized the same BG505 SOSIP construct and further experimented with mixing and matching antibodies. The original PGT122 Fab provided good lattice contacts with the BG505 Env trimer, but the resolution and anisotropy of the diffraction was not ideal. Addition of a newly discovered antibody

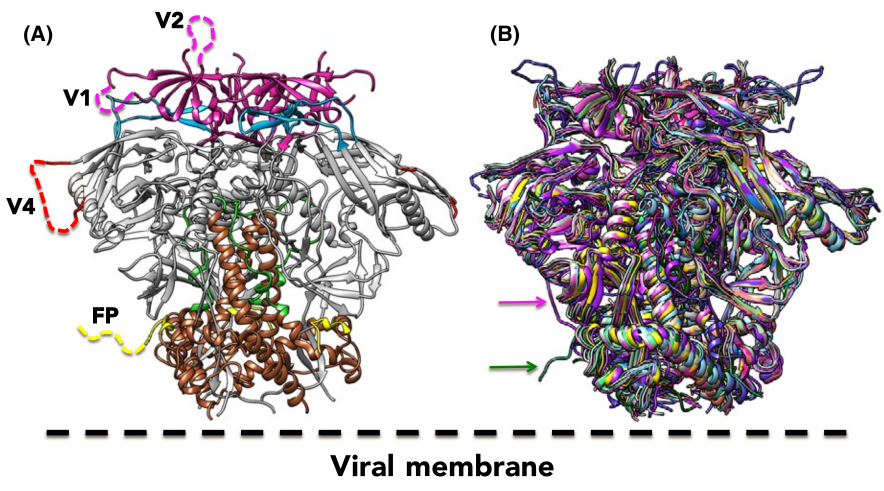

FIGURE 1 The Env trimer architecture. (A) The 3 Å crystal structure of BG505 SOSIP Env (PDB 5CEZ). Different regions of the trimer described in the main text are colored accordingly: gp120 (gray), V1/V2 trimer apex (magenta), V3 loop (cyan), V4 loop (red), gp41 (brown), N-terminal region of HR1 ($HR1_N$) (green), and fusion peptide (yellow). Dashed lines indicate the locations of flexible, typically disordered regions in the trimer structures. The $HR1_N$ region (green) is also usually disordered in SOSIP structures, although fully resolved in 5CEZ. This same region in native Env trimers lacking the SOSIP mutations adopts a helical topology (PDB 5FUU). (B) Overlay of all Env trimer structures to date determined by cryo-EM and x-ray crystallography at <6 Å resolution (PDB: 3J5M, 4NCO, 4TVP, 4ZMJ, 5ACO, 5C7K, 5CEZ, 5CJX, 5D9Q, 5FUU, 5FYJ, 5FYK, 5FYL, 5I8H, 5JS9, 5JSA) demonstrates the conserved fold of the trimer, with small differences in the variable loops at the periphery of the trimer. The arrows indicate the fusion peptides from two different structures (5FUU, magenta and 5I8H, green) demonstrating its highly flexible nature

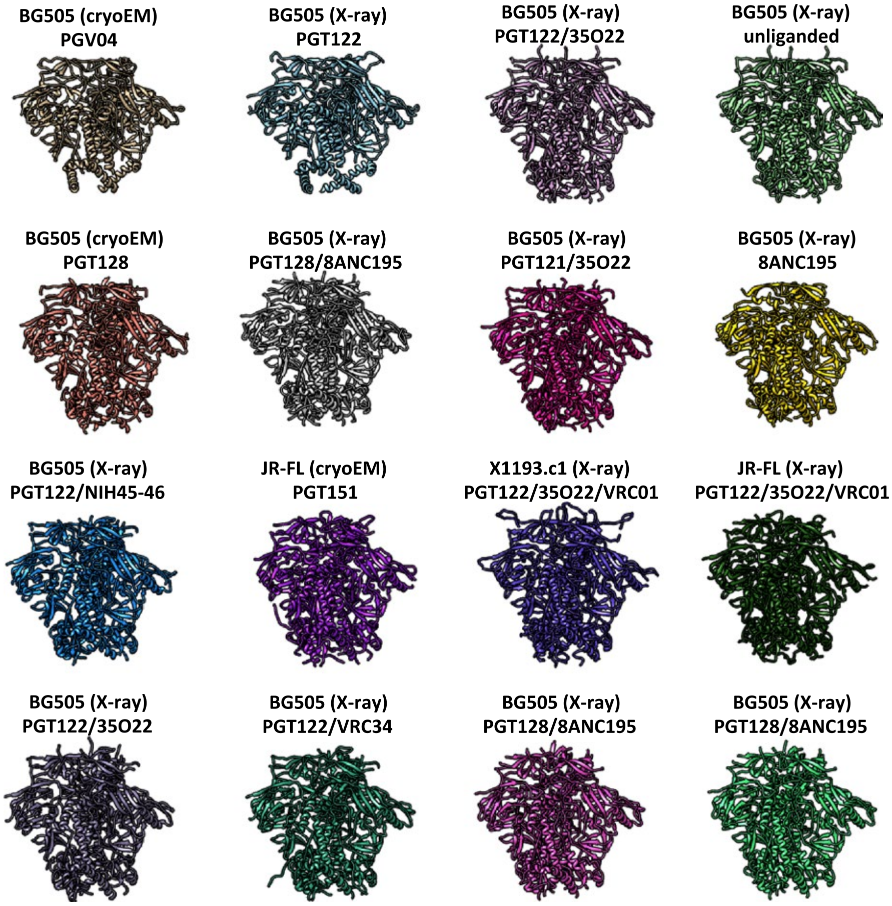

FIGURE 2 Overview of Env trimer structures solved to date at <6 Å resolution arranged in order of their structures being determined. First row: (PDB: 3J5M, 4NCO, 4TVP, 4ZMJ), Second row: (PDB: 5ACO, 5C7K, 5CEZ, 5CJX), Third row: (PDB: 5D9Q, 5FUU, 5FYJ, 5FYK), Fourth row: (PDB: 5FYL, 5I8H, 5JS9, 5JSA). The headers above the structures indicate the strain (clade A BG505, clade B JR-FL, or clade G X1193.c1), the technique used to solve the structure (cryoEM or X-ray), and the antibodies bound in the deposited structure (the Fabs are not shown for clarity). Most of the crystal structures have an antibody that targets the N332 supersite in gp120 (PGT122 or PGT128) and a gp41 or gp41/gp120 base-binding antibody (35O22, 8ANC195, or VRC34), which also aid in crystallization. Antibodies can induce local structural changes within and proximal to their epitopes, particularly in the variable loops (V1-V5), the fusion peptide, and glycans (data not shown). Some of these subtle differences are obvious in the images in the figure

35O22 from Mark Connors laboratory led to crystals that increased the resolution to approximately 3.5 Å, although still with considerable anisotropy.[59] This more complete structure of the Env trimer revealed fascinating new details of the prefusion gp41 conformation including the SOS disulfide, the intricate arrangement of the N- and C- terminal regions of gp120 with gp41 at the base of the trimer, and a tryptophan clasp that locked the fusion peptide proximal region in its prefusion conformation (Figures 1 and 2).

In our lab, we also continued to explore different combinations of antibodies to search for that elusive Env trimer crystal that diffracted well in all directions (i.e. isotropic diffraction). Combination of different antibodies led to various structures of BG505 SOSIP with PGT122 and Fab NIH45-46[60] at 4.4 Å, with PGT128 plus 8ANC195 at 4.6 Å,[61] with 8ANC195 at 3.6 Å,[62] and finally with Fabs 3H+109L (a putative heavy chain precursor of the PGT121 lineage) and 35022 to achieve 3.0 Å resolution where fully isotropic diffraction was observed from these crystals.[63] This latter structure completed the description of gp41, including the largely disordered region around the top of the central HR1 helices ($HR1_N$) that connect to the fusion peptide proximal region (FPPR) and which contains the I559P mutation that stabilizes the prefusion conformation of the SOSIP trimer. Notwithstanding, this region is still very flexible in the soluble trimers but is less so in native, almost full-length trimers as discussed below. In practice, this flexible region has also become a target for engineering alternate trimers, also discussed below. Furthermore, these new structures with potential precursors and intermediates of the heavy chain of the highly potent PGT121 lineage have provided valuable information on the evolution of different branches of an antibody lineage and how alternate solutions can arise even starting from a single unmutated common ancestor.[63,64]

4 | HIV-1 GLYCOPROTEIN STRUCTURES—THE GLYCAN SHIELD

The glycan shield on HIV-1 has long been thought to act as an impenetrable barrier to the immune system. The 80-90 glycans that cover the surface of the HIV-1 Envelope protein (Env) are put on and processed by our own cells in the journey of the Env glycoprotein from the endoplasmic reticulum through the Golgi to the cell surface and then into the virion itself. These self-glycans should protect the virus against an immune response through tolerance mechanisms, but the virus has gone overboard in almost completely covering the Env surface making it unlike any glycoprotein on the surface of our own cells or even on most other viral glycoproteins (Figure 4). So the burning questions that percolated for several years were: what does the glycan shield look like, what is its exact composition, and what is its three-dimensional structure. Hints came from x-ray structures of the high mannose patch targeting PGT128 bound to a minimal gp120 outer domain construct[65] and V1V2 targeting PG9 bound to a scaffolded

V1V2 domain.[66] Both structures demonstrated how antibodies used long CDRH3 loops to penetrate the glycan shield, albeit in a minimal monomeric context.

The extensive glycosylation on HIV-1 was therefore somewhat of an enigma. On soluble BG505 SOSIP Env, 81 potential N-glycosylation sites are encoded by Asn-X-Ser/Thr motifs (27 per monomer) and almost all of them appear to be occupied. This high density of glycans of the HIV-1 surface led to the notion of it being a protective coat against antibodies in the immune system. To circumvent some of the heterogeneity in the glycans for structural studies, the Env proteins were expressed in human embryonic kidney (HEK) 293S GnTI$^{-/-}$ cells that produce high mannose glycans and no complex sugars. The glycans were then trimmed with the endoglycosidase EndoH—glycans that are protected within the three-dimensional structure or are proximal to the bound bnAbs are not cleaved by EndoH and can often be more readily visualized. The first X-ray trimer structure showed an extensive assortment of glycans covering the surface [see figure 1 of (14)] particularly around the high mannose patch and around the glycopeptide epitopes bound by the bnAbs. Further x-ray structures of BG505 SOSIP with different antibodies have reinforced this view that the ordered glycans are either closely clustered on the surface or interact with bnAbs. Indeed, a glycan at position 262 is particularly well ordered as it is intimately involved with the folding of gp120 and also helps buttress other glycans on the surface of Env.[61]

However, in the examples above, the glycans in the x-ray structures are much more homogeneous than on native virions due to expression in cell types such as mammalian HEK 293S cells that restrict the glycoforms to high mannose and which then can be partially trimmed by EndoH. One major advantage of electron microscopy is that the sample protein does not have to be crystallized. Hence, heterogeneous glycosylation can be accommodated in EM structure determination. The cryo-EM samples were therefore produced in HEK 293T cells with glycosylation profiles similar to that on the virus itself. Indeed, the first cryo-EM structure of the BG505 SOSIP trimer showed a great variety of glycans on the Env surface [see figure S4 of (13)].

It became clear that EM had a major advantage for sample preparation and that the full complement of glycans, high mannose, hybrid and complex, can be present on the specimen for structure determination. Notwithstanding, the crystal lattice contacts of the BG505 SOSIP trimer with PGT122 and 35022 were made almost entirely by the Fabs and, therefore, provided an unexpected opportunity for fully glycosylated Env to potentially be visualized in the electron density maps of crystal structures. The Kwong group further modified the Env trimer complex by engineering in cysteine mutations in the gp120 and in the bound scFv or Fab VRC01 Fab (G459C in gp120 and A60C in the VRC01 heavy chain), that enabled crystallization of fully glycosylated trimers (high mannose) not only of clade A BG505 SOSIP, but also from clade G (X1193.c1 SOSIP.664) and clade B (JR-FLSOSIP.664)[67] (Figures 1 and 2).

Perhaps unexpectedly, a much more extensive and highly ordered glycan shield was visualized consisting of 29 of the 31 high mannose glycans on the clade G Env with similar outcomes for clade A and B trimers. Three types of glycan-glycan interactions were observed that depended on the distance between glycans and the comparative density of the surrounding glycans. Closer distances between neighboring glycans splayed them apart, whereas intermediate distances allowed them to be more upright and interact at their tips, much like the canopy of a dense forest. Longer inter-glycan distances did not permit such ordered glycan-glycan interactions and, hence, fewer sugar moieties were observed at such sites. Such an extensive and ordered network of interactions appeared to fly in the face of carbohydrate modeling on the Env surface, where they were previously observed to rapidly flicker as if on off hot coals.[35, 68]

Prior to the structures mentioned above, cryo-EM was also again used to visualize the glycan shield- this time with all native glycans that include complex, hybrid and high mannose glycoforms[69, 70]—at atomic resolution. One of these, an almost full-length native structure was determined by Jeong Hyun Lee in the Ward lab using cryo-EM with no stabilizing mutations (Figure 3)—the membrane proximal external region (MPER) and membrane-spanning region were intact, and only the cytoplasmic tail was deleted from the construct.[69] Extensive complex tri- and tetra- antennary sugars were seen in gp41, particularly those that interacted with the PGT151 bnAb that was used to extract and stabilize the trimer from membranes, but not the extensive glycan canopy in gp120 seen in the x-ray structures.

How do we resolve such differences in the glycans observed in these x-ray and EM structures? In the crystal structures, the glycans are homogeneous, being all high mannose. In the EM structure, the glycans are much more diverse and heterogeneous and would lead to fewer glycans being visualized as multiple glycoforms are present at many positions, especially those that mainly consist of complex and hybrid glycans.[71] But, for the high mannose patch on gp120, which should be relatively homogeneous in both,[71–73] fewer sugars and glycan interactions were seen in the EM structure. Natural glycan processing may to some extent account for this as it depends on the entire constellation of glycans surrounding each and every glycan- even the high mannose patch has complex or hybrid sugars interspersed between and surrounding the high mannose clusters that may make them more heterogeneous in the native versus high mannose glycoproteins. Completely homogenous high mannose sugars may also have a greater tendency to cluster and form defined interactions, but that has still to be verified. The x-ray structures were also complexed with antibodies that interact with and help order glycans as noted in previous x-ray[14, 59, 63] and EM[13] studies. In the x-ray structures,[67] two antibodies are bound to gp120 and one to gp41, whereas in the cryo-EM structure,[69] only one antibody PGT151 is bound asymmetrically (and only two per trimer) at the gp120/gp41 interface. Thus, nine antibodies are bound per trimer in the x-ray structure and only two per trimer in the EM structure. Notably, the most ordered glycans in gp120 in the x-ray structure, as well as the complex glycans in gp41 in the cryo-EM structure, are proximal to where the antibodies are bound. A further x-ray structure of BG505 SOSIP with three 8ANC195$_{G52K5}$ Fabs bound per trimer also showed highly ordered high mannose glycans surrounding the bound antibody.[62] Thus, it is now well-appreciated that, when antibodies bind to their glycopeptide epitopes, they can help order the glycans that are part of or proximal

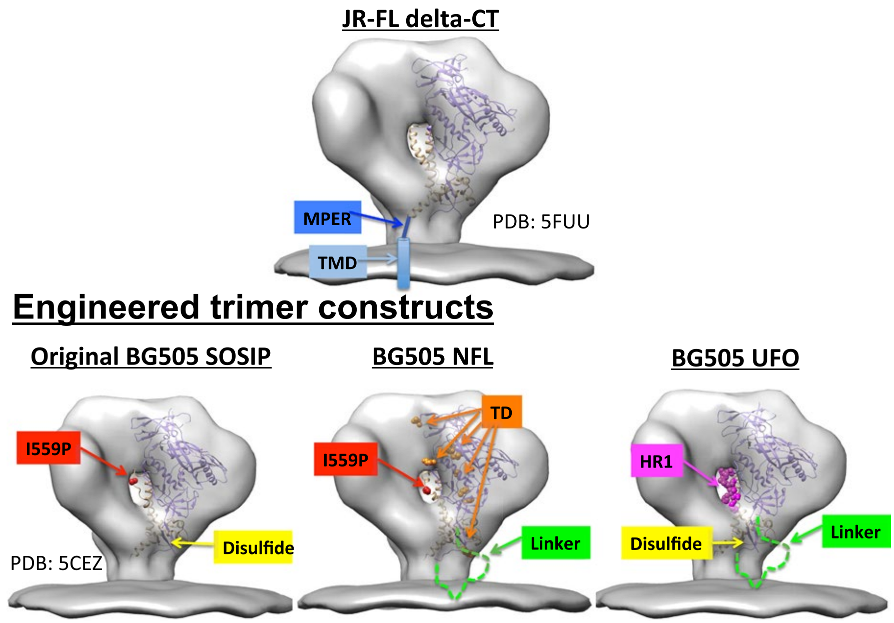

FIGURE 3 Full-length and soluble Env constructs. The almost complete native trimer with membrane-proximal external region (MPER) and transmembrane domain (TMD) with only the C-terminal domain deleted (ΔCT) is superimposed on a low resolution cryo-ET reconstruction of Env on a viral membrane (EMD-5019). The soluble cleaved BG505 trimer is shown on the bottom left with the SOSIP mutations. The NFL trimer construct is shown in the middle with a linker replacing the cleavage sequence between gp120 and gp41 with the I559P mutation form SOSIP and other trimer-domain mutations added to expand the variety of strains and clades that can use this platform.[84, 85] A further uncleaved UFO construct has a truncation and modification of $HR1_N$ as well as the SOS disulfide and a flexible linker region connecting gp120 and gp41 (bottom right)[21]

to the epitopes. Finally, it might it possible that crystallization can select out more ordered glycoforms, or the crystallization buffer and low pH may artificially promote glycan-glycan interaction.

Notwithstanding, a great swath of supple sugars, numbering around 1000, covers the HIV Env surface and forms a protective glycocalyx[74] that gently sways in the breeze and masks the Env protein surface beneath (Figure 4). Despite this shimmering sugar sheath, antibodies can in time, usually over a period around 2 years or more, evolve to insert between the glycans using rapier-like long CDRs that can attack the more vulnerable conserved regions of the Env protein below. Thus, most of these sites of vulnerability, or epitopes, on the Env trimer are composed of both glycans and amino acids from the Env protein. An increasing number of sites where antibodies can strike have been found, but these antibodies come too late in natural infection. The question now is how to use all of this exciting structural information to help design a vaccine that takes into account the complex

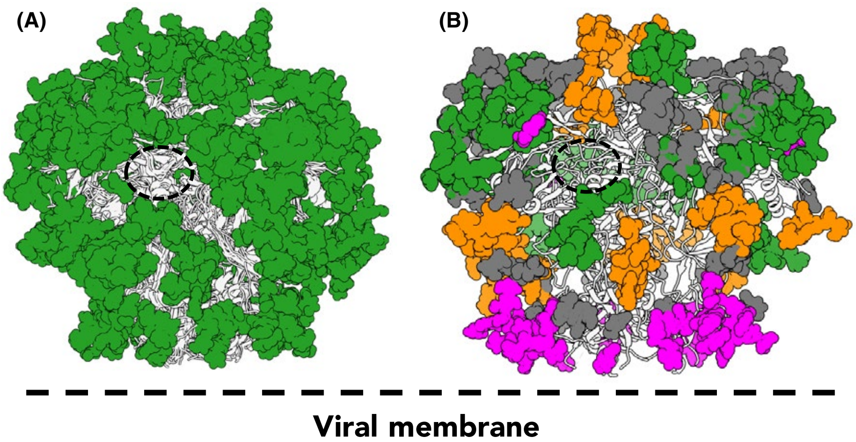

FIGURE 4 (A) Superposition of Env trimer structures determined to date with all resolved glycans colored green. While much of the surface of Env is covered by glycans, a few peptide epitopes are still accessible, most notably the CD4 binding site (dashed oval), although access is still highly restricted by the surrounding glycans. (B) The glycans resolved in the BG505 (PDB 2FYL) and JR-FL (PBD 5FUU and 2FYK) Env trimers are displayed and colored according to the glycan site-specific analyses of BG505 SOSIP Env in[71] (high mannose: green, complex: magenta, high mannose and complex: orange, unknown: gray)

nature and diversity of the glycan shield and that can more rapidly elicit antibodies that protect against HIV infection.

An outstanding question that remained is the exact composition of the glycans or the glycoforms at individual glycosylation sites within the Env trimer (Figure 4). In order to construct more complete models of the Env trimer than include the glycan shield, it is essential to know what are the specific glycans or collection of glycans that can be built into the x-ray electron density or the EM reconstructions. It has been known for some time that a high level of glycan homogeneity existed in a region known as the high mannose patch[75–77] (Figure 4), due to steric crowding that impeded secondary processing from high mannose sugars to more complex glycoforms by glycosidases.[73, 75] This secondary processing takes place in the golgi after the protein is fully folded and hence reflects either the capability or inability of glycosidases to access their glycan substrates on the Env protein [see figure 4 of (72)]. Thus, the tightly crowded high mannose patch on the outer surface of gp120 is essentially independent of the cell line used for protein expression.[73] In gp41, as the glycan sites are further apart (Figure 4), more complex or hybrid glycans predominate and the glycosylation then becomes cell-line dependent due to the differential activities of glycosidases in different cell lines.[73] Another important feature is that cleavage of the trimer is critical in attaining proper native-like glycosylation—uncleaved trimers have more processed glycoforms[73] indicating that structure is not folded into the closed native conformation.[47] Indeed, non-native uncleaved gp120 proteins also contain aberrant disulfide bonds.[78]

Thus, a substantial breakthrough was required to quantitate the glycan composition at individual glycosylation sites and that was achieved recently by using mass spectrometry to analyze the glycoforms on the Env trimer.[71] The site-specific glycosylation separated into sites that were essentially all high mannose, those that were complex, and other site where mixtures of glycoform types were present. The high mannose patch extends like a belt around the central region of gp120 on the trimer and the more complex forms are mainly located at the trimer apex and the gp41 base with mixed forms scattered more sparsely throughout (Figure 4). Thus, this new glycan information is invaluable for not only constructing the best models of Env glycoproteins but also for design of immunogens that more accurately mimic the viral Env.

5 | SOLUBLE SOSIP TRIMERS FROM OTHER CLADES

After finding a soluble cleaved SOSIP trimer (BG505) that was suitable for both structural and immunological studies, it was not clear how generally applicable this SOSIP platform would be for other strains of HIV-1. Over the past 3 years, several other Env proteins from diverse subtypes have been expressed as SOSIP proteins. Clade B and C trimers as well as other clade A trimers based on the SOSIP platform have been designed[79–81] and indeed have been used effectively as immunogens to elicit autologous tier 2 neutralizing antibody responses in animal models.[82] The Kwong group has similarly used this platform to construct trimers from clades B and G.[67] Thus, the SOSIP trimer platform has now been successfully applied to many different strains and subtypes (Figures 1 and 2) and thus is of general utility for producing soluble Env trimers for structural and vaccines studies.

6 | ALTERNATE HIV-1 ENV TRIMER PLATFORMS

Other platforms have also been explored for the production of Env trimers (Figure 3). We will not discuss here the expression of uncleaved trimers containing foldon and Leu-zipper domains as these trimers do not fold into native-like configurations.[47, 83] We will focus on more recent platforms where native-like Env conformations have been obtained using different designs. Two of these designs modify the cleavage site between gp120 and gp41 (Figure 3). One of these designs, termed native flexibly linked (NFL), replaces the natural cleavage sequence REKR with Gly_4-Ser linkers between the natural C- terminus of gp120 and N-terminus of gp41,[84] where two such repeats were optimal. This more flexible linker, which also increases the distance between the normal C-terminus of gp120 and N-terminus of gp41, appears to allow the uncleaved trimers on BG505 and JR-FL backbone to achieve native-like configurations in the absence of cleavage by furin. However, the I559P mutation that was necessary for stabilization of SOSIP trimers was also required here. This NFL platform has now been used to express Env proteins for other B and C subtypes by incorporating residues from BG505 that are important for trimer formation [called trimer-derived residues (TD)].[85] A similar design also experimented with inserting Gly-Ser linkers in place of the cleavage sequence on a BG505 backbone where a linker of 15 residues gave optimal properties. This construct maintained the SOSIP mutations (SOS: A501C-T605C and IP: I559P).[86] Thus, these designs resulted in soluble Env trimers that did not need furin for cleavage to achieve native-like conformations and, hence, could potentially simplify the expression of Env trimers.

Using the available Env trimer structures, further efforts to stabilize the soluble Env trimer for immunogen design have been undertaken. These efforts have been quite successful at increasing the melting temperature of trimers by up to approximately 20°C. A variety of mutations have now been introduced to stabilize the closed prefusion conformation of the Env trimer[87] and also engineering a disulfide into the bridging sheet to prevent the conformational change to the CD4 bound form.[33, 85]

Another recent design has focused on redesigning the largely disordered loop ($HR1_N$) in the soluble Env constructs connecting the central HR1 helix to the FPPR.[88] Stable trimers that express well were obtained using a designed shorter $HR1_N$ region that was used for production of native-like structures. Further replacement of the cleavage site, as with the designs above, with both short and long linkers led to another platform that can be used to produce soluble Env trimers, called UFO trimers (Figure 3), in high purity and yield for different HIV strains and subtypes.

7 | STRUCTURAL PLASTICITY

We have focused so far on ways to make soluble, stable Env trimers for structural, immunological and vaccine studies. However, viral fusion glycoproteins in general are metastable and undergo complex conformational rearrangements in transitioning from the prefusion form that binds receptor to the postfusion form that is able to fuse the viral membrane with the host cell membrane to transfer the viral genome into the cell for replication. However, it appears that the HIV-1 Env protein is more unstable than its counterpart on influenza virus, where its envelope glycoprotein (hemagglutinin) can be readily cleaved from the virus[2, 6] or expressed as a soluble, highly stable protein.[6] The RSV protein is also quite unstable and readily adopts a postfusion form even on the surface of the virus before cell entry.[15]

Recent studies from Walther Mothes and colleagues using single-molecule fluorescence resonance energy transfer (FRET) have vividly demonstrated that unliganded HIV Env is intrinsically dynamic and that it flickers between three distinct conformation on the viral surface.[89, 90] Binding of bnAbs, as well as the natural receptor CD4, can influence the relative distribution of these conformations.[89] Thus, it is not surprising that the recombinant forms of the Env protein also have similar but dampened dynamic character, as they are inherent to the protein itself and not on the engineering or expression system used. This flexibility manifests itself in the reactivity under some conditions (e.g. ELISA) of soluble SOSIP trimers with V3 antibodies where it appears that slight opening of the apex can expose the highly immunogenic V3 loop.[46] Indeed, different strains and subtypes appear to vary in the extent to which they are fully closed.[80] We have called these forms closed and partially open as observed by negative–stain EM and we routinely assess each soluble Env construct and preparation for closed, partially open, and open configurations, where the first two forms are native-like.[80]

It has been also observed that antibodies are not always present in full stoichiometry (i.e. 3 per trimer) when bound to the Env trimer. Glycan heterogeneity may partially explain this observation[13] as well as induced conformational changes in the trimer after antibody binding. In the full-length cryo-EM structure at approximately 4.2 Å resolution with the intact transmembrane domain, some subtle conformational differences are observed in the presence of antibody PGT151, which binds at the gp120/gp41 interface and holds the trimer together to enable its extraction from membranes.[69, 91] PGT151 binding to two equivalent sites in the Env trimer appears to pry open the subunit interface at the third site rendering it unavailable for binding to a third antibody and further highlighting the structural plasticity of the Env trimer. In fact, soluble BG505 SOSIP retains this plasticity and also only binds one or two antibodies per trimer, despite the stabilizing mutations.[91] The full-length structure also has a less well-resolved trimer apex, consistent with flexibility in V1/V2. The disordered region at the top of the long HR1 central helix ($HR1_N$) connecting the fusion peptide region is however much more ordered and largely helical in the wildtype structure, further emphasizing the key role of the I559P mutation in the soluble SOSIP trimer in destabilizing the postfusion conformation by disrupting the helical propensity of this $HR1_N$ region. This structure also contained the MPER, and binding of MPER bnAb 4E10 showed that the MPER was likely extracted from the membrane upon antibody binding resulting in an apparent elevation of the trimer off the membrane surface, further demonstrating the deformable nature of the trimer.

8 | FUSION PEPTIDE

In other viral proteins such as influenza hemagglutinin, the fusion peptide (FP) is buried deeply between subunits within the trimer core.[6] The fusion peptide is a highly conserved sequence at the N-terminus of gp41 that is liberated after furin cleavage of gp160. The fusion peptide was not observed in the initial soluble Env protein structures, but the location of the fusion peptide proximal region suggested that it might indeed be close to the viral surface. In most SOSIP structures, the first 5-10 residues of the FP point toward the solvent and are disordered. Unlike other type I fusion viral glycoproteins, the FP appears to point away from the core of the trimer, extending past HR2 and toward glycans at N611 and N637. The full fusion peptide has recently been resolved but only in the presence of bnAbs (PGT151 and VRC34) that target this region (Figures 1 and 2). The fusion peptide is fully exposed in these structures in the interface between the antibody and the Env surface and constitutes a major component of the epitopes.[69,92] Thus, somewhat unexpectedly, the fusion peptide is accessible to antibodies and has now become yet another target for HIV vaccine design.

9 | EPITOPES OF BNABS AND GLYCAN HOLES

The highly glycosylated surface of Env presents a barrier that bnAbs must negotiate to access the peptide surface, either by directly binding the glycans or avoiding them as much as possible.[63] Thus, all of the epitopes except 2G12 are comprised of a mixture of peptide and glycan elements [reviewed (48)]. The available peptide surface on Env outside of the CD4bs is typically very small and most bnAbs have long CDR H3 loops that can navigate through the glycan shield and contact the underlying protein residues to access an epitope composed of both glycan and peptide residues. Recently, immunization of rabbits with the BG505 SOSIP trimer resulted in a neutralizing antibody response that was directed to a breach in the glycan shield where glycans were not present in BG505 as in some other strains and subtypes.[93] BG505 has three glycans that are absent compared to more glycosylated Env proteins and the antibodies seem to be able to access this "glycan hole" more readily.[93] This observation suggests that the immune response prefers to target accessible peptide surface and that the glycans act as a largely impenetrable barrier for some time after infection. In fact, even highly potent and evolved bnAbs typically exhibit a relatively slow on rate of binding that is likely attributable to negotiating the glycan shield.

In contrast with these limited observations to date for vaccine-induced neutralizing antibodies, bnAbs elicited from HIV-1 infected individuals target multitude of epitopes that now decorate almost the entire surface of Env. This phenomenon is also in stark contrast to influenza virus, where the main epitopes are functional and constitute the receptor-binding site and the stem fusion domain [reviewed (94–96)]. Thus, the constant interplay between evolving virus(es) within an individual after infection, and the months to years before bnAbs evolve, perhaps allows the immune system more time to target many other sites in addition to the receptor binding site and fusion domain. Many broadly neutralizing antibodies also target the high mannose patch, which because of the homogeneity in glycans, is more conserved than one might have imagined given normal glycan heterogeneity. Some of the antibodies also appear to be effective in stabilizing prefusion versions of the Env trimer and thereby prevent the conformational changes and rearrangements associated with receptor and co-receptor binding and fusion.[97]

10 | CONCLUSIONS AND FUTURE PERSPECTIVES

It is remarkable that, in the short space of 3 years, 16 x-ray and cryo-EM structures of Env trimers from 3 to 6 Å resolution have been determined given that no atomic-level structures existed before 2013. A combination of different factors contributed to these recent successes and included the critical development of a stable soluble trimer that was native-like and amenable for high-resolution structural studies.[46] An equally important component for structure determination was the diverse assortment of human broadly neutralizing antibodies that became increasingly available after 2008.[48–54] The Fabs from these antibodies could be exploited in a combinatorial manner using high-throughput crystallization screens with Env trimers, and robotic systems [e.g. see (98)] to improve the odds of forming a crystal lattice that minimized contacts with the heterogeneous glycosylation of the Env trimer and could produce crystals that diffract to high resolution. These antibodies were also not only important for crystallization, but also for providing appropriate mass and features for single particle cryo-EM. Furthermore, these antibody complexes have elucidated the structure and features of the broadly neutralizing epitopes in the context of the trimer and accelerated the design of immunogens as vaccine candidates. The soluble Env trimers from the BG505 strain have provided the majority of the structures determined to date, but this SOSIP platform has turned out to be transferable to other strains and subtypes (Figures 1 and 2). These breakthroughs inspired the design of other platforms that capitalized on the stabilizing mutations that proved critical in the SOSIP constructs (Figure 3). While it became clear that uncleaved trimers did not fold into compact trimers,[47] two groups explored replacing the cleavage site with a linker that would provide more flexibility and enable the uncleaved trimers to adopt a native-like prefusion trimer configuration[84–86] (Figure 3). The original I559P mutation that was essential to stabilize the SOSIP trimers is in a highly flexible loop that zips up into a helical structure in the postfusion form after receptor and co-receptor binding. This proline mutation reduced the propensity for such a conformational change. In another design effort, this loop was assumed to be a major cause of the trimer metastability-thus, the $HR1_N$ loop was shortened and modified in sequence to prevent such a helical postfusion conformation and, when combined with addition of a flexible linker between gp120 and gp41, led to a platform that produced good yields of soluble trimers for a diverse array of strains and subtypes[88] (Figure 3). Thus, within a short span of 2–3 years, new Env trimers were produced on different backbones with different platforms and with increased stability that clearly illustrated that such modifications did not perturb the conserved Env trimer fold and could accommodate the regions of sequence and structural hypervariability (Figures 1 and 2).

The next major breakthrough came from determining the structure of a native trimer without any stabilizing mutations. Antibody PGT151 can stabilize native trimers on membranes[91] that enables the native trimer to be extracted for structural studies. The cryo-EM structure of an almost full-length JR-FL native trimer with MPER and the transmembrane domain, but without the cytoplasmic domain, was determined[69] and then compared to the soluble Env protein (Figures 1 and 2). While subtle differences were seen in the apex and in the FPPR, the overall fold was very similar for the native and soluble trimers that put to rest any lingering questions about differences in soluble versus native trimers. What the glycan shield looked like was also addressed in these studies (Figure 4) as well as site-specific information on the composition of the glycoforms at individual glycosylation sites.[71–73] Thus, we now have an excellent and graphic representation of the glycans covering and protecting the Env protein surface from these structural and functional analyses (Figure 4). The myriad of complexes with diverse antibodies has also uncovered a much greater number of broadly neutralizing epitopes than expected and provided invaluable information for design of immunogens as vaccine candidates.

What then is next? As structural biologists, we are always trying to achieve the highest resolution structures to provide a better understanding of structure and function. Pushing the resolution to around 2 Å will also aid substantially in immunogen design. So achieving better crystals and, in particular, utilizing the enormous advances in cryo-EM will undoubtedly enable the current 3 Å barrier to broken. Different functional forms of the trimer such as with CD4 receptor or CCR5 co-receptor as high resolution will also provide invaluable information on how this metastable trimer transitions through prefusion to intermediate to fusion-active forms. Design of germline trimers that can kick start the immune response along paths known to produce bnAbs in HIV-infected individuals may also be instrumental in arriving at better vaccine candidates. Thus, while it has taken time, structural information on the HIV Env trimer is now coming at an impressive pace and should markedly accelerate the search for the elusive HIV vaccine.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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