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Structure-based vaccine design in HIV: blind men and the elephant?

Robert Pejchal¹ and Ian A. Wilson^{1,2,*}

¹ Department of Molecular Biology, The Scripps Research Institute, La Jolla, CA 92037 USA

² Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, CA 92037 USA

Abstract

Traditional vaccine approaches have failed for HIV and novel strategies are now being sought to develop immunogens designed to elicit specific activity against known broad neutralization epitopes. Structure-based vaccine design has great potential but, thus far, remains a largely unproven concept. Further structural information for the envelope (Env) glycoproteins, gp120 and gp41, is needed, particularly for understanding trimer-specific antibodies and their epitopes and to clarify atomic details of the structural elements responsible for masking crucial epitopes and for mediating the conformational rearrangements undertaken during the process of receptor-binding and membrane fusion.

Introduction

Vaccines are most effective against pathogens for which natural infection elicits a robust antibody (B cell) and/or cellular (T cell) immune response [1]. In such cases, exemplified by influenza and polio, a live-attenuated or killed preparation is sufficient to confer protection against infection or contain the virus, if infection does occur. However, for HIV, no cases are known of natural clearance and, furthermore, the virus rapidly establishes reservoirs (through integration and latency) that are resistant to even the most aggressive anti-retroviral treatment (HAART, highly active anti-retroviral therapy). Thus HIV presents unique problems that will likely require a vaccine that confers sterilizing or close to sterilizing immunity (antibody-mediated) and rapid and robust cytotoxic T-lymphocyte-mediated elimination of newly infected cells [2]. Barriers to the production of such a vaccine include the well-documented ability of HIV to mutate rapidly, and, thus, escape antibody and T-cell responses, and extensive glycosylation of Env, which reduces or impedes recognition of protein surfaces by neutralizing antibodies. Poor immunogenicity and instability of the native viral envelope glycoproteins, combined with antibody responses to non-neutralizing epitopes elicited by immunodominant regions of non-native forms of gp120 and gp41, further contribute to this problem. However, it has been shown recently that broadly neutralizing antibody responses against Env develop in a larger percentage of HIV-infected individuals than previously thought [3–8], but they do so slowly. Importantly, animal studies demonstrate complete protection by passive immunization with such antibodies [9,10]. Thus, hopes have been raised that, if the right immunogen is found, it may, indeed, be effective in conferring immunity to HIV [11].

The story of the blind men and the elephant, a well-known Indian tale about three blind men asked to describe an elephant wherein each draws a different conclusion based on touching a

* Address correspondence to this author at, Department of Molecular Biology, The Scripps Research Institute, La Jolla, CA 92037 USA; wilson@scripps.edu.

different part of the animal, seems particularly relevant to structure-based vaccine design efforts in HIV. The idea that an understanding of one part engenders a sufficient understanding of the whole, and that meaningful and accurate extrapolations may be derived from such limited information, is both a strength and weakness of the current scientific methods that can be employed for vaccine design. In the case of structure-based immunogen design for HIV, several monoclonal antibodies (mAbs) are now known that recognize neutralizing epitopes that appear to be worthy targets for vaccine development. Such antibodies are able to recognize a wide range of primary isolates and are thus termed broadly neutralizing antibodies (bNAbs). Analysis of these bNAbs has established the presence of several distinct neutralizing epitopes in gp120, gp41, and the intact Env trimer. However, a high-resolution crystal structure of the intact gp120/gp41 trimer has been extremely difficult to determine. Thus, a complete understanding of the neutralizing epitope landscape and the glycoprotein elements and conformational changes governing access to these critical regions is lacking. What is known structurally about the interaction of bNAbs with their epitopes must, so far, be considered largely in the context of gp120 or gp41 fragment crystal structures or from lower resolution cryo-electron tomography (cryo-ET) studies of native trimers [12]. Therefore, important parameters of neutralization, such as epitope exposure and appropriate angle of approach of the antibody to its epitope on the Env trimer on the viral or cell surface, must be inferred.

An important question facing the HIV-vaccine design community is whether an Env glycoprotein trimer crystal structure is absolutely essential or enough is known already to enable the successful design of immunogens aimed at targeting the known neutralization sites. Available structures exist only for truncated and deglycosylated gp120 core monomers, and mainly in a single conformation (the CD4-bound state), although antibody-bound and binding site mimic structures are available, as well as an unliganded, glycosylated SIV gp120 structure [13]. However, shed monomer and uncleaved gp160 are known to induce mainly non-neutralizing antibodies and it is unclear during natural infection whether neutralizing antibodies can be elicited by such viral debris [14]. Therefore, in the absence of an Env trimer crystal structure, can enough information be gleaned from fitting gp120 core structures in complex with bNAbs into cryo-ET reconstructions?

This review then will focus on describing what has been learned from available structures and explore whether the structural information is sufficient to allow the design of viable, structure-based immunogens in light of the recent isolation of extremely potent and broadly neutralizing antibodies. Given that directed affinity maturation of neutralizing antibodies that recognize complex, discontinuous surfaces, or continuous, linear surfaces, likely necessitates that immunogens stably display such epitopes [15], lack of an atomic-level trimer structure will continue to compound the challenges faced in HIV structure-based design, particularly for neutralizing epitopes that have not yet been structurally defined.

HIV-1 Env structure: do we know enough?

gp120

The first gp120 crystal structure was determined in 1998 and is a landmark in the HIV-1 field [16]. In order to produce diffraction-quality crystals, it was necessary to truncate the N- and C-termini, referred to as the C1 and C5 (constant) regions (Fig. 1) denoting their conservation across isolates. In addition, the hypervariable V1/V2 and V3 loops were removed by genetic truncation and replaced by Gly-Ala-Gly linkers [17]. Finally, the residual glycans on gp120 were removed enzymatically and the resulting deglycosylated “core” was complexed with a soluble, two-domain version of CD4, CD4(D1D2), and the Fab from CD4-induced (CD4i) antibody Fab 17b. The addition of these ligands stabilized the conformation of gp120 and the newly acquired, non-glycosylated, more rigid protein

surfaces allowed formation of well-ordered crystals [17]. Core gp120 retains the ability to bind CD4 and properly display CD4-induced epitopes, indicating functional relevance. Structures with complete N- and C-termini [18] and V3 loops [19,20] have since been completed using the same strategy of CD4 and CD4i antibody co-crystallization. However, the degree of protein engineering and ligand-induced stabilization required to solve these structures is unusual and may predict challenges for the determination of full-length glycosylated gp120 monomer and gp120/gp41 trimer structures.

The gp120 structure consists of a highly-glycosylated, double β -barrel outer domain, an inner domain that includes a 3-helix, 4-strand bundle, a 7-stranded β -sandwich, and a 4-strand “bridging sheet” composed of two strands from the inner domain (the V1/V2 stem) and two strands from the outer domain (β 20- β 21) (Fig. 1) [18]. The structure does not resemble any other known protein structure. CD4 binds in a depression between the inner and outer domain and the bridging sheet, which is thought to form stably only after binding of CD4 [16,21,22]. The presence of a “Phe43 cavity” and other structural analyses [16], together with large entropic changes associated with binding of CD4 to both core and full-length constructs [23,24], may be taken as evidence for CD4 induction of the observed conformation that assembles the bridging sheet. Indeed, the conformation of elements that compose the bridging sheet vary widely among the currently available gp120 structures, which include complexes with non-neutralizing CD4bs antibodies, F105 and b13 [25], as well as bNAb b12 [26], and differ substantially from the CD4-bound structures (Fig. 1). In the b12-bound state, the β 20- β 21 hairpin is an extension of β -strands 19 and 22, helix α 1 is partially unwound, compared with the CD4-bound state, and the V1/V2 stem is largely disordered. In the b13-bound state, β 20 morphs into a short helix separated from residues that compose β 21, and the V1/V2 stem is freely mobile and able to participate in crystal contact formation. These differences exist despite the presence of most of the same stabilizing mutations (designed to promote the CD4-bound state) in the gp120 cores used to obtain both complex structures. Furthermore, non-neutralizing CD4bs mAbs recognize conformations of these regions that are thought to be incompatible with the native trimer [25]. In contrast, the footprint of b12 on gp120 is centered more on the outer domain and farther away from the bridging sheet [26], and binding does not induce such a large-scale restructuring [23]. Thus, binding of b12 is compatible with the gp120 conformations occurring on the oligomeric spike, as has been observed [12]. Importantly, despite 12 years since the initial structure determination of core gp120, the b12-bound structure, until recently, was the only atomic description of a bNAb in complex with a relatively complete (non-peptide or carbohydrate) antigen. An exciting new addition to this structural repertoire is the VRC01 bNAb complex with HIV-1 gp120 core in which the heavy chain is a good mimic of CD4 itself [27].

Conformational instability of gp120 likely promotes elicitation of non-neutralizing CD4bs Abs, and may partly explain why subunit vaccines based on gp120 monomers are not effective (reviewed in [28], also see [29,30]). Furthermore, gp120 may also sample conformations that have yet to be structurally characterized [16,31–34]. Binding of CD4 also increases access to V2 and V3 epitopes, likely by inducing large-scale reorientation of V1/V2 and liberating V3. Structures of full-length, glycosylated gp120 are now needed in order to fully understand the architecture of gp120, including V1/V2 and V3, in the pre-receptor bound state.

gp41

The transmembrane glycoprotein (gp41) drives membrane fusion using a mechanism common among enveloped viruses [35,36] and similar to cellular membrane fusion machinery [37]. Functional elements of gp41 include the N-terminal fusion peptide (FP), liberated by furin cleavage of the gp160 precursor, the N-terminal and C-terminal heptad repeat regions (NHR and CHR, respectively) connected by a disulfide-bonded loop, the

membrane proximal external region (MPER), and the transmembrane (TM) spanning region (Fig. 1). During later stages of receptor binding, the fusion peptide embeds into the target cell membrane, while the TM region remains in the viral membrane. The MPER appears to play a fusion pore-related role during close apposition of the viral and cellular membranes, and is required for productive fusion [38]. Following triggering by CD4 and CCR5, one of the multiple coreceptors capable of being used by HIV-1, gp41 undergoes a series of refolding events that ultimately lead to formation of the six-helix bundle, post-fusion conformation, with the fusion peptide and TM region present in the merged membrane [39].

Much can be gleaned from the sequence similarity of gp41 to other viral membrane fusion proteins. The six-helix bundle resembles similar post-fusion structures for influenza virus [40], ebola virus [41], visna virus [42], human respiratory syncytial virus [43], and human T-lymphotropic virus type 1 [44]. Where structures for pre-fusion and post-fusion states are available, conformational rearrangements that approach almost complete refolding of the fusion domain are observed; influenza HA2 [45], ebola gp2 [46], and parainfluenza virus 5 F2 [47] refold substantially, and post-fusion structures are not particularly informative of the conformation of the pre-fusion state. Thus, little can be said about the pre-fusion state of gp41 in the absence of a complete (pre-fusion) gp120/gp41 trimer.

Env trimer tomography

No crystal structure of a retroviral envelope trimer has yet been determined. Nevertheless, cryo-ET or single particle cryo-electron microscopy reconstructions are available for several retroviral Env spikes, including Moloney murine leukemia [48], human foamy virus [49], and more recently for SIV [50,51] and HIV [12]. The first studies of SIV mac239 trimers were in conflict and neither could be fit unambiguously with existing crystal structures [50,51]. The recent determinations of a native HIV-1 trimer, based on the BaL isolate, led to reconstructions that could be cross-validated with crystal structures of gp120 in complex with soluble CD4 and 17b Fab, as well as with b12 Fab [12], providing increased confidence that the resulting analyses are free of processing artifacts or bias. Cryo-ET reconstructions of three forms of the trimer have been characterized; free (unliganded), b12-bound, and CD4/17b-bound (Fig. 2). Various conformational states are now experimentally observed from these reconstructions that go from closed to intermediate (b12-bound) to open (CD4/17b-bound). With this limited survey of possible ligands, it is difficult to generalize at present, but it is clear that, in this case, substantial conformational rearrangements of the trimer accompany binding of both b12 and CD4 and lead to stable populations of open forms. Crystal structures of gp120 fitted to the reconstructed maps revealed large-scale conformational rearrangements in both the orientation and rotation of gp120 with respect to gp41 and the trimer axis upon binding of CD4. The reconstructions allow mapping of the known neutralizing epitopes on the functional trimer (Fig. 2). Furthermore, useful models and distance measurements may be derived from modeling and analysis, and such studies suggest that steric occlusion of both the CD4bs and MPER deter the elicitation of bNAbs against these neutralization epitopes [52].

The closed conformation of the trimer shows clear separation of the gp120 protomers, with a prominent reduction in density at the geometric center of the spike (Fig. 2) [12]. At low resolution, both the CD4-bound and b12-bound gp120 structures produce equally acceptable fits to the trimer density. However, the CD4-bound conformation of gp120 would orient the V1/V2 loop orthogonal to the trimer axis, where there is no apparent density to accommodate the bulky domain, and, therefore, the variable loops are inferred to be localized at the apex of the spike. In the intermediate state, the gp120 protomers appear to expand to accommodate the bound Fab b12, however the V1/V2 and V3 loop stems are still oriented toward the apex, where significant unmodeled density is also found. Only the variable loops and gp41, then, would be expected to contribute to interactions at the trimer

interface. In the open state, the trimer undertakes significant conformational rearrangements when CD4 and Fab 17b are bound that involve both rotation and movement of gp120. Strikingly, the gp120 protomers move apart and gp120-gp120 interactions are broken, presumably together with formation of the bridging sheet, giving a dramatic lateral swinging of the V1/V2 domain, for which unmodeled density adjoining 17b is seen. Conformational changes are also evident in gp41, although no appropriate structures are available for fitting.

Although the cryo-ET reconstructions have not elucidated any new atomic details, the emerging picture of a native spike at low resolution has already brought many important new insights. The spike is conformationally flexible, accommodating the relatively bulky b12 substituent and undergoing large-scale reorientations of the three gp120s in the trimer, likely concomitant with changes induced in gp120 itself. Modeling of the glycans reveals that the CD4bs is the only significantly exposed protein region on the otherwise highly masked surface of the native trimer [52]. However, although the locations of the variable loops and gp41 can be inferred, a detailed understanding is precluded by lack of availability of crystal structures representing these states. Most importantly, no trimer contacts between gp120 can be identified even with the most complete gp120 model available [18]. Thus, structural descriptions of the missing regions of gp120 and gp41 and further analysis of the different conformational states of the Env trimer and their relative propensities in tier 1 and tier 2 viruses are badly needed.

Broadly neutralizing antibodies: targets for retrovaccinology

CD4 binding site: b12, HJ16, VRC01

The CD4 binding site (CD4bs) is the site of attachment for the primary receptor. It is functionally conserved and must remain exposed for the virus to latch on to target cells. Three known CD4bs bNAbs have been characterized: b12 [53], HJ16 [54], and VRC01 [55]. The breadth and potency of b12 and HJ16 are similar; b12 neutralizes nearly 50% of isolates [54,56,57], although it is mainly limited to clades B, C, and D [56], while HJ16 neutralizes 36% of viruses in the same panel [54]. VRC01 is broader and more potent than b12, neutralizing about 90% of isolates screened [55], and may represent a general template for structure-based vaccine design efforts[58].

The wide coverage of isolates neutralized by bNAbs recognizing the CD4bs makes this epitope an ideal target for vaccine design; however, subunit vaccines based on gp120 have thus far failed to elicit these types of antibodies [29,59–61]. Strategies aimed at improving gp120 immunogens to better display the epitopes recognized by CD4bs-directed bNAbs include hyperglycosylation [62], mutation [63,64], stabilization [26,65,66] (resurfaced stabilized cores [55]), and truncations to produce the outer domain that constitutes the majority of the CD4 and b12 bindings sites [67]. These strategies also typically utilize the deletion of hypervariable loops, which are immunodominant [33,34].

Hyperglycosylation of gp120 resulted in a variant gp120 that retained b12 recognition, but limited the binding of non-neutralizing CD4bs mAbs, and was a significant technical achievement given the lack of a b12-bound gp120 crystal structure to aid design [64,68](n.b. these concepts were also later applied to gp140 [69]). Stabilization of the CD4-bound state is another strategy, specifically designed to limit the conformational flexibility between the inner and outer domains [26]. Using the structure as a guide, Dey *et al.* tested gp120 antigens containing “cavity-filling” mutations to reinforce hydrophobic cores and engineered disulfides to minimize flexibility and promote sampling of the CD4-bound state [65]. The resulting antigens showed quite different immunogenicity and antigenicity compared with parental gp120 core constructs, demonstrating that conformational fixation can, indeed, impact these immunological parameters, although the stabilized constructs

elicited CD4i antibodies in preference to CD4bs [65], as previously observed for immunization with stable gp120-CD4 complexes [70]. In a recent study, resurfaced stabilized gp120 core proteins that selectively interact with b12, but not with the majority of non-neutralizing CD4bs mAbs were created [55]. These engineered proteins have their non-essential, exposed residues mutated to SIV or non-HIV residues and, as such, are also not recognized by mAbs outside the CD4bs. Thus, they have potential to focus the immune response on a more narrowly defined CD4bs epitope if used as the boost component in a prime-boost strategy. So far, none of these strategies has yet elicited neutralizing CD4bs antibodies with sufficient potency and activity against tier 2 viruses, which are more neutralization resistant and considered representative of primary circulating isolates. As elicitation of non-neutralizing (or poorly neutralizing or weakly cross-reactive) CD4bs Abs occurs with much greater frequency [56,65,71], better gp120 monomer immunogens and immunization strategies are needed. Understanding the steric barriers to CD4bs access on neutralization-resistant (tier 2) viruses and the conformation and location of V1/V2, β 20- β 21, and V3 loop segments in the unliganded state, should inform the design of next generation gp120 immunogens.

High-mannose glycan cluster: 2G12

Although many viruses rely on host ‘self’ glycans to shield neutralizing epitopes, hyperglycosylation of gp120 gives rise to a sterically-occluded glycan patch that is incompletely processed by host mannosidases. This array of high-mannose glycans (including Asn295 and Asn332) is recognized by the bNAbs 2G12 [72], the only known exclusively anti-carbohydrate bNAbs to HIV. Glycans required for 2G12 recognition are relatively conserved in subtype A and B viruses, but not on subtype C and CRF01_AE viruses, which 2G12 typically does not neutralize [56]. Although protein-carbohydrate interactions are typically weak, 2G12 uses a novel domain exchange arrangement to yield an Fab dimer that forms an extended, multivalent binding surface composed of two conventional antigen combining sites and a third potential binding site at the novel $V_H/V_{H'}$ interface [73], thus gaining greater avidity.

Given the conservation of many of the glycans found on gp120, particularly high-mannose sugars, hyperglycosylation is clearly important for structure, antigenicity, and viral fitness [74]. Recent work on re-eliciting 2G12-like antibodies includes investigation of the antigenicity of a yeast protein, PstI, produced in a yeast strain that confers high-mannose glycans of Man₈ length on the protein surface [75,76]. Immunization with the mutant yeast strain efficiently elicits antibodies that recognize the immature α (1,2)-linked mannose residues, and the resulting sera contain antibodies that recognize both HIV and SIV gp120 [77]. Numerous other synthetic carbohydrate immunogens (reviewed in [78]) have been tested, but most give rise to sera that do not efficiently recognize gp120. A crucial question for anti-carbohydrate vaccine design is whether domain-swapping is required for high-affinity binding to the high-mannose cluster on the outer domain, or whether avidity can be gained through cross-linking gp120 protomers within the spike [52]. Some progress on the number of mutations required for domain swapping has been made [79], as well as the use of modified sugars as better potential immunogens [80].

Membrane proximal external region: 2F5, Z13e1, 4E10

The membrane proximal external region (MPER) of gp41 is situated between the C-heptad repeat region and the transmembrane region. This highly-conserved, Trp-rich region is important for productive membrane fusion, and is also the target of three bNAbs, 2F5, Z13e1, and 4E10, which have different fine specificities, potencies, and breadth [81–83]. It has been suggested that the MPER epitopes do not become fully exposed until after CD4-triggering of the spike [84], although binding to unliganded trimers has also been observed

[85,86]. As the MPER-directed bNAbs are the only antibodies that are known to block post-CD4 attachment [87], they are thought to neutralize by interfering with a late stage transition in gp41 refolding required for membrane fusion.

2F5 recognizes a minimal epitope that includes residues E⁶⁶²LDKWAS⁶⁶⁸, whereas 4E10 recognizes a downstream sequence W⁶⁷²FDITNWLW⁶⁸⁰. The 2F5 epitope conformation is largely extended leading up to the D⁶⁶⁴KW⁶⁶⁶β-turn that is buried within the combining site [88,89]; however, thereafter the peptide epitope has significant helical character [90,91]. 4E10 recognizes a fully helical epitope conformation, embedding W⁶⁷²F⁶⁷³ in the combining site. Interestingly, these residues are thought to insert deeply into the viral membrane, interacting with the acyl chain portion of membrane lipids [91], so it is unclear how 4E10 can bind to its epitope on an intact, membrane-embedded Env trimer. In addition, the neutralizing activities of 2F5 and 4E10 have been shown to rely on interaction with the viral membrane, a property that is separable from recognition of the protein antigen [92,93]. For 4E10, recognition of membrane can be mapped to specific hydrophobic residues within CDR H3 [92]. Z13e1, the least potent of the three antibodies [94], recognizes an epitope between 2F5 and 4E10, W⁶⁷⁰NWFDITN⁶⁷⁷, which forms a two-helix, elbow-like structure (with a hinge region) and a hydrophobic face opposite to the antibody combining site [95]. The epitope that overlaps that of 4E10 is visualized in a distinctly different conformation when bound to Z13e1 [91], indicating a conformational change can occur within the MPER that utilizes the ability of Asn to act as a helix-capping residue [95]. Thus, the discrepancy between conformations recognized by Z13e1 and 4E10 may stem from their recognition of different states of the MPER, or different binding modes, for example MPER extraction from the membrane by 2F5 and 4E10 [91,96]. Such a mechanism may explain the requirement of 2F5 and 4E10 to bind the viral membrane.

Serum specificity studies have shown that anti-MPER activity is not very common and, when present, rarely represents the dominant neutralizing activity [4–8,54,97–102]. On the other hand, elicitation of such antibodies with designed immunogens is conceptually more straightforward due to their linear nature (most neutralizing epitopes are discontinuous) and the availability of high-resolution characterization of the relevant, antibody-reactive MPER conformations. Attempts to re-elicite such neutralizing activity have thus far failed [103,104], and a growing appreciation of the importance of long, hydrophobic CDR H3s that have affinity for lipid argues for lipid-embedded presentation of the MPER immunogens. However, structural characterization of the interaction of antibodies with membrane is lacking and may be challenging given the lack of stable, membrane-embedded, native trimer molecules with which to co-crystallize the bNAbs. Studies of membrane interfacial proteins may aid the design of gp41 display scaffolds that orient appropriately in lipid [105].

The presence of the MPER in any trimeric immunogen may be a complicating factor, potentially contributing to aggregation. Technologies, such as lipid nanodisks [106], are being explored to minimize the non-specific interactions of MPER immunogens with host cellular membranes. Other methods, such as virus-like particles [86,107], may be of use if they can be produced without incorporation of host proteins. Arrayed antigens, such as displayed MPER on non-polymorphic viral surfaces [108,109], may overcome low natural abundance. Finally, 2F5 and 4E10 have been shown to protect rhesus macaques against challenge with SHIV(Ba-L) [110], offering continued optimism for development of MPER immunogens.

Conserved regions of the V1/V2 and V3 loops: PG9/PG16

Hypervariable loop regions V1/V2 and V3 have overall higher mutations rates than other regions within Env, yet also contain well-conserved clusters that are functionally important [111]. One such region within V2, N¹⁵⁶CSFNITTSIRDK¹⁶⁸, is the target of two V2-directed

mAbs, C108g [112] and 10/76b [113]. C108g, isolated from a chimpanzee infected with the HXB2 isolate, is very potent against the parental strain and BaL, but its neutralization is otherwise very restricted. 10/76b also shows very narrow cross-reactivity and has somewhat weaker potency compared with C108g [113]. Human antibody 2909 [114], which binds elements of V2 and V3, recognizes only the trimeric form of Env as presented on viral or cell surfaces [115]. Although 2909 is also very type-restricted, this mAb is extremely potent. The narrow specificity of 2909 derives from its dependence on a relatively rare residue, Lys160, normally a glycosylated Asn residue found in over 93% of all isolates. Thus, some epitopes within the hypervariable loops are exposed on trimers and can mediate potent neutralization

PG9 and PG16, isolated from a single donor infected with a clade A strain, differ by somatic variation and recognize a V2/V3 epitope preferentially displayed on cell surface trimer [57]. Both are extraordinarily potent and among the most broadly neutralizing antibodies isolated to date, but differ in their epitope fine specificity and potency against certain strains. The most pronounced difference between the two mAbs is PG16's reliance on V3, which is not seen for PG9, except near the V3 base [57]. In addition, both are dependent on the highly conserved, glycosylated Asn160 and neutralization is strongly compromised by mutation of Asn160->Lys [57]. Certain residues that are associated with a PG9 and PG16 knock-down phenotype also increase sensitivity to other neutralizing antibodies [57,116–120], which may indicate that they affect the integrity of the unliganded state of the native Env trimer. The combined breadth and potency of PG9 and PG16 and their recognition of an epitope that is exposed on the unliganded spike make them ideal candidates for a vaccine.

The recently determined crystal structure of PG16 revealed that its unusually long CDR H3 (28 residues) forms a compact subdomain that protrudes a long way from the antibody combining site [121,122]. The hammerhead structure also reveals that, unexpectedly, both PG9 and PG16 have sulfated tyrosine residues [121], a modification previously seen only in CD4i antibodies [123]. Although PG9 and PG16 differ from CD4i antibodies in that they compete with soluble CD4 and are dependent on V2 and V3 for neutralization, they may be able to position their H3 subdomains to interact with some part of the recessed coreceptor binding site on the unliganded trimer, taking advantage of the sulfophilicity of gp120 to boost binding affinity.

Importantly, PG9 and PG16 react poorly with recombinant Env monomer and trimer and were identified by functional (microneutralization) assays [57]. Although both bind very well to cell surface trimers, they bind weakly to soluble trimer immunogens, such as YU2 gp140-foldon [124] and KNH1144 SOSIP [125,126], that are constructs engineered from isolates that are potentially neutralized. Interestingly, PG9 shows weak binding to recombinant gp120 monomers from certain isolates, exemplified by DU422. The structural basis for this reactivity is not understood; however, it is believed that PG9 and PG16 do not recognize a truly quaternary epitope, as mixed trimer experiments suggest binding does not require more than one WT gp120 protomer within the spike [57].

The identification of PG9 and PG16 illustrates the limitations of gp120 core structures; gp120/gp41 trimer and full-length gp120 monomer structures are essential to provide an atomic description of the epitope to which PG9 and PG16 bind. Without such structures, it is not possible to directly design stabilized immunogens that optimally express the PG9/16 epitope. In the absence of a stable, native trimeric immunogen, attempts at re-eliciting PG9 and PG16 like antibodies may have to rely on DNA-based approaches, a relatively recent strategy that is not yet represented in licensed vaccines.

Strategies for production of soluble, stable trimers

A compelling case may be made for using recombinant trimeric antigens over gp120 monomer as the protein component of a vaccine. The native trimeric Env complex presents epitopes for broadly neutralizing antibodies, including CD4bs and PG9/16 epitopes, and does not expose non-neutralizing epitopes. The efficacy of soluble recombinant influenza HA, which induces protective antibodies that compare favorably with traditional vaccines [127,128], together with findings that HA trimers are more effective than matched monomers [129], represent proof of concept for using HIV Env trimers to achieve these same goals. Indeed, the traditional inactivated influenza vaccine is so effective that it is now largely a manufacturing problem, limited only by the timely incorporation of seasonal (and regional) sequences and the emergence of pandemic strains. Thus, a search for epitopes that may induce bNAbs is now ongoing in order to produce a “universal” vaccine. The recent identification of one such epitope, the membrane proximal (HA2) stem [130,131], provides a critical test for structure-based vaccine design. Typically robust antibody responses combined with recognition of the HA2 stem by near-germline (V_H1–69) antibodies [132,133] provide optimism for the utilization of this region for the creation of a universal vaccine. Recent findings that stem antibodies can be elicited by vaccination [134] may represent encouraging progress toward a more universal flu vaccine.

The instability of the native gp120/gp41 trimer, however, presents challenges for evaluating their efficacy in vaccine trials. Flexibility of the molecule is functionally important; the trimer undergoes large conformational changes during the two-step entry process and must also evade antibody responses by rapid mutation and conformational masking. The tendency of trimer to dissociate and the absence of a naturally occurring disulfide bond between gp120 and gp41, such as is naturally present between influenza HA1-HA2 and ebola gp1-gp2, also distinguish HIV Env from these other viral fusion proteins. One means to overcome some of this instability has been the introduction of an engineered disulfide between gp120 and gp41, termed an “SOS” linkage [135]. Moreover, a mutation in HR1, I559P, has been identified that also stabilizes the non-covalent association of SOS gp140 in trimer [136]. The combination of these approaches yields “SOSIP” gp140 trimers. Alternative designs for the generation of stable trimers have included the use of heterologous trimerizing sequences together with mutagenic disruption of the furin cleavage site [124,137,138]. The expression of unmodified ectodomains results in more heterogeneous monomeric and oligomeric forms, some stabilized by disulfide bonds [139] that are likely non-native [140]. Several such oligomeric gp140 proteins have been assessed in immunogenicity studies [141–144] (reviewed in [142]). Although the neutralizing antibody responses have been marginally, though in many cases reproducibly, better than gp120 monomers, no study has succeeded in generating high titer neutralizing sera with activity against the harder to neutralize tier 2 panel of HIV viruses. Presumably, the current generation of soluble gp140 trimer immunogens still suffers from excessive flexibility, conformational heterogeneity, and non-native presentation of conserved neutralizing epitopes.

Trimerization by itself, then, is not enough to produce completely native trimer. One possible reason is that gp120 packing within recombinant soluble trimers is not optimal, with the outcome that significant non-neutralizing (and immunodominant) surfaces are still well exposed. Evidence for this defect comes from observation of the binding of non-neutralizing antibodies to these soluble trimers, as well as lack of recognition by PG9 and PG16 [57]. The role of gp160 cleavage by furin on antigenicity also remains unclear. While cleavage is known to affect the presentation of neutralizing epitopes [145], and has been shown to improve gp41 antigenicity in a soluble trimer format [146], uncleaved gp160 on the cell surface binds PG9 and PG16 comparably to mature, cleaved Env [57]. However, the availability of trimer-specific bNAbs, like PG9 and PG16, now gives immunogen designers

the critically needed metrics to establish improved antigenicity criteria for gp120 in next generation soluble trimers. Recognition of PG9 and PG16 is not only a necessary prerequisite to improving antigenicity, it is likely to reduce the immunodominance of less relevant regions exposed on “floppy” trimers. For example, V3 is the major immunodominant region of gp120 and readily elicits antibody responses. Yet V3-directed antibodies rarely neutralize tier 2 viruses, which typically occlude V3 prior to binding of CD4, and exhibit limited cross-reactivity. The crystal structure of the unliganded state of the Env trimer will reveal how the variable loops are situated and possibly interlock at the apex of the trimer, and make possible the use of atomic details to engineer mutations designed to stabilize antigenically native, soluble trimers.

Ideal properties for a trimeric immunogen would include native antigenicity and minimization of non-neutralizing epitope surfaces by oligomeric occlusion. Stability, both in terms of gp120/gp41 association and gp140 association in trimer, lack of aggregation, homogenous glycosylation, and proper multivalency are also desirable properties. Trimers should present symmetric and conformationally homogenous epitopes, including quaternary epitopes, to allow saturation of possible binding sites by antibodies. Different strategies to allow conformational stabilization of either the unliganded or CD4-triggered states, providing two relevant and very distinct epitope landscapes, may also be useful for elicitation of alternate specificities. For example, a closed state is likely required for elicitation of PG9/16-like antibodies while an open state might better elicit MPER-directed bNAbs.

Closing thoughts

Of the known neutralization specificities, epitopes consisting of the CD4bs and conserved variable loop regions, recognized by PG9 and PG16, appear to be the most promising. Recent studies have clearly demonstrated that CD4bs specificity is well represented in neutralizing sera. New serum mapping studies suggest that the prevalence of the PG9/16 specificity among HIV-1 infected patients with broad and potent neutralizing sera may be as high as 21% [102]; therefore, a strong case for pursuing vaccine strategies to re-elicite their activity may be made. The elicitation of antibodies against epitopes that are normally occluded by glycan is not likely to confer protection; however, the high-mannose sugars can also be targeted by antibodies. Novel potent and broad specificities will likely be found, and structural immunology will continue to prove an invaluable tool for understanding the neutralizing epitopes and designing strategies for their re-elicitation.

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Figure 1. Structures of gp120

(A) Crystal structure of deglycosylated gp120 core monomer with complete inner domain in complex with CD4(D1D2) and Fab 48d (ligands not shown); PDB 3JWD [18]. The inner domain (grey), outer domain (orange), and bridging sheet (yellow), composed of the V1/V2 stem and β 20- β 21 hairpin, are the main structural features found in the CD4-liganded conformation. The V1/V2 and V3 loops are truncated. Asp368, which interacts with Arg59 from CD4, is depicted as red sticks. (B) Crystal structure of gp120 core with intact V3, bound to CD4(D1D2) (pale yellow) and the Fab of sulfated CD4i antibody 412d (light chain pink, heavy chain red); PDB 2QAD [20]. (C) b12 gp120 complex structure. Colors are same as in panel A, b12 light chain (cyan) and heavy chain (blue); PDB 2NY7 [26]. (D) b13 gp120 complex crystal structure, b13 light chain (light green), b13 heavy chain (dark green); PDB 3IDX [25].

Figure 2. Trimer cryoelectron tomographic reconstruction density

(left)Side-view of cryo-ET density for an unliganded BaL trimer that is fitted with the crystal structure of a b12-bound gp120 core monomer. The locations of epitopes for bNAbs are shown.(top right panel)Unliganded trimer density fitted with the b12-bound gp120 core monomer (left), b12-bound trimer density fitted with b12-bound gp120 monomer in complex with b12 Fab (middle), CD4/17b-bound trimer density fitted withCD4/17b-bound gp120 core monomer in complex with CD4(D1D2) and 17b Fab[12].These three reconstructions represent the closed, intermediate and open states of the trimer. (bottom right panel) Same structures as top, but from an overhead view. Coloring scheme: gp120: green, Fab: cyan (light chain) and dark blue (heavy chain), and CD4 (yellow). Tomographic reconstruction densities and crystal structures fitted to the density were rendered in UCSF Chimera [147].