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Nat Rev Immunol. Author manuscript; available in PMC 2011 September 6.

Published in final edited form as:
Nat Rev Immunol. 2010 July ; 10(7): 527–535. doi:10.1038/nri2801.

STRUCTURE–FUNCTION RELATIONSHIPS OF HIV-1 ENVELOPE SEQUENCE-VARIABLE REGIONS PROVIDE A PARADIGM FOR VACCINE DESIGN

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Abstract

One of the main challenges of developing an HIV-1 vaccine lies in eliciting immune responses that can overcome the antigenic variability exhibited by HIV. Most HIV vaccine development has focused on inducing immunity to conserved regions of the HIV envelope; however, new studies of the sequence-variable regions of the HIV-1 gp120 envelope glycoprotein have shown that there are conserved immunological and structural features in these regions. Recombinant immunogens that include these features may provide the means to address the antigenic diversity of HIV-1 and induce protective antibodies that can prevent infection with HIV-1.

The gp120 and gp41 glycoproteins of the HIV envelope (Env) are targets of antibodies that inhibit virus infectivity, and attempts to induce these antibodies with a prophylactic HIV vaccine have used various approaches. The failure of early gp120 protein-based vaccines to induce neutralizing antibodies[1] redirected attention to the cellular arm of the immune response. The subsequent failure of a T cell-based vaccine[2] left the HIV-1 vaccine field at a crossroads, with the direction forward uncertain. Recently, a dual component HIV-1 vaccine, in which priming with a recombinant canarypox vector was followed by boosts with two recombinant gp120 proteins, imparted a measure of protection from infection[3]. Protection in this trial may have been due to antibodies[4], but the nature and specificity of the protective antibodies, and how to design immunogens that induce higher and more persistent levels of protection remains unresolved.

The difficulties of HIV-1 vaccine research are, in part, a result of its extreme antigenic variation. Conventional wisdom suggests that "constant" rather than "variable" regions of Env should be targeted to elicit broad responses against antigenically diverse HIV strains. However these regions were classified in early studies on the basis of the sequences of only a few virus strains[5]. Immunological and 3D structural studies of the Env of diverse strains have now revealed higher order structural features that would alter these early classifications and explain how antibodies specific for some variable regions have neutralization activity for diverse viruses. Although several epitopes that induce neutralizing antibodies have been identified in conserved regions of both the gp120 and gp41 portions of Env, inducing neutralizing antibodies that target these epitopes using rationally designed immunogens has, so far, proven difficult[6–9]. Moreover, although a vigorous antibody response is induced by infection, only a minority of individual HIV^+ patient sera neutralize a broad spectrum of HIV strains[10,11].

We believe that targeting conserved elements within the immunogenic sequence-variable Env regions (Figure 1) using rationally designed immunogens is promising, but inducing

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broad, cross-strain neutralizing antibodies specific for these regions is a challenge. Nevertheless, current evidence suggests that 3D visualization might identify invariant epitopes capable of inducing protective antibodies that are hidden within the sequence variable Env regions. Indeed, this perspective provides a rational basis for understanding the documented immunological cross-reactivity of many monoclonal antibodies targeting the second (V2) and third (V3) variable loops of gp120, and the quaternary neutralization epitopes (QNEs) formed by V2 and V3 (Box 1 and Table 1). In hindsight, this perspective is a logical outgrowth of classic immunochemical studies[12] showing that cross-reactive antibodies recognize antigens that are related but not identical in sequence (as for example with anti-V2 monoclonal antibodies that recognize gp120 monomers derived from diverse primary isolates of HIV-1[13]). These old but seminal studies support a new paradigm for HIV-1 immunogen design.

In this article, we synthesize data from both the biological arena (viral and immunological studies) and the physical arena (structural and bioinformatics studies) to show how structure–function relationships of Env proteins can inform the design of an effective HIV vaccine. With special emphasis on the variable regions of gp120, we highlight the relationship between 3D molecular structure, biological function and immunological cross-reactivity. This approach demonstrates the potential utility of targeting sequence-variable regions and QNEs that are formed by the interaction of sequence-variable regions in the context of the Env trimers. Because of their sequence variability, these regions were previously dismissed as vaccine targets, but we now think that they should be reassessed as potentially valuable targets.

Biological functions of the various gp120 regions

Antibodies that interfere with key biological functions of the virus are able to “neutralize” virus infectivity. Therefore, understanding the function of various parts of Env assists in targeting antibodies that should be induced by protective vaccines.

Receptor-binding sites

It is now well-established that to trigger exposure of the HIV-1 gp41 fusion domain that initiates virus–cell fusion, gp120 must first bind to two cell-surface receptors: CD4 and CXC-chemokine receptor 4 (CXCR4) or CC-chemokine receptor 5 (CCR5)[14,15]. The receptor-binding surfaces on gp120 form distinct structural and antigenic regions that are highly conformational and are formed by discontinuous amino acids in several regions of gp120[16,17](Box 1). The CD4 binding site (CD4bs) consists of many residues in the constant regions of gp120, but, until recently, only one of many CD4bs-specific monoclonal antibodies had been described with broad neutralizing activity[18]. Three more have recently been selected and characterized[19,20](Table 1). This suggests that the critical region within the large CD4bs may be difficult to target with vaccine constructs, a fact that is supported by disappointing attempts to induce neutralizing CD4bs-specific antibodies with various vaccine constructs [6,7,9].

The chemokine receptor-binding region of gp120 consists of the invariant β2, β3, β20 and β21 strands (the bridging sheet), the V3 loop, and the stem of the V1/V2 double loop[16,17]. This highly conformational, discontinuous region of gp120 is often known as the CD4-induced (CD4i) region, because, as its name implies, it is formed after gp120 has bound CD4 with high affinity. Many human monoclonal antibodies specific for CD4i have been described[21–24], indicating the strong immunogenicity of this region, but CD4i, when exposed on the virus particle, is sterically protected from most CD4i-specific antibodies during the process of infection owing presumably to its close proximity to the cell

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membrane[25]. Consequently, CD4i-specific antibodies may not protect against infection by most HIV-1 strains.

The V3 loop

The V3 loop of gp120 is a component of the chemokine receptor-binding region, and is a major determinant of co-receptor usage[26–31]. The length of the V3 loop is essentially constant at 34–35 amino acids. Although its amino acid sequence is highly variable, except in clade C viruses where its sequence is quite conserved[32], sequence variation is restricted to only ~20% of the amino acid positions, and is located primarily in the two β-strands in the crown of the V3 loop (Figure 1B **and** 2D). The functional importance of V3 is illustrated by the fact that deletion of V3 completely abrogates virus infectivity[33]. Nonetheless, the value of V3-specific antibodies in blocking virus infection has been controversial because some V3 antibodies, especially those elicited early after infection, have limited breadth[34], many neutralize only the most neutralization-sensitive “Tier 1” viruses[19,35], no single V3-specific monoclonal antibody neutralizes more than ~10–25% of the more resistant “Tier 2” viruses[19,36,37], and the accessibility of V3 is poor on many viruses[38,39]. However, given its essential function in HIV infectivity, its structural conservation[40,41], its strong immunogenicity[42], the existence of many V3-specific monoclonal antibodies with cross-clade neutralizing activity [19,36,37,43], and the demonstration that polyclonal V3-specific antibodies can be induced that neutralize viruses from diverse HIV-1 subtypes[44], the V3 region remains a potentially important target for vaccine development.

The V2 loop

By contrast, V2 is not essential for infectivity[33], which suggests that the evolutionary constraints to preserve an invariant V2 structure are less pronounced than for V3. However, V2 participates in several Env functions, providing the motif that binds to α4β7 integrin (the receptor apparently involved in the homing of the virus to gut mucosal cells)[45], contributing to trimer formation [46], and participating as part of the chemokine receptor surface. V2 also plays a crucial role in masking neutralizing epitopes of gp120: changes in V2 length and glycosylation patterns allow viruses to escape from neutralization mediated by antibodies specific for V3 and the CD4bs of gp120[47–50]. Given these functions of the V2 loop, its newly recognized role as a component of QNEs[51–54] and its partial structural conservation (see below), V2 becomes an additional target for vaccine development.

V1, V4 and V5

The functions of the other variable regions of gp120 are poorly defined. V1 may have functional significance as it can serve as a neutralizing epitope, and several potent neutralizing V1-specific monoclonal antibodies have been developed in transgenic mice[55]. The immunogenicity of V1 is affected by glycosylation[56] and mutations in V1 can affect virus-induced syncytium formation[57], but little more than this is known about its function. V4, by contrast, has no well-defined function, although it is a target for early autologous neutralizing antibodies[58], and V4-specific neutralizing antibodies have been described in immunized rabbits[59]. V4 is involved in neutralization escape[60], and undergoes extreme variation in early infection[61]. The V5 region, the only variable region that does not form a disulphide-linked loop, participates in forming the CD4bs surface, but no polyclonal or monoclonal neutralizing V5-specific antibodies have been described; nonetheless V5 is involved in neutralization escape, although V5 peptides were not able to block neutralizing antibody activity in sera[62]. Current data, therefore, do not support these variable regions as targets for HIV-1 antibody-based vaccines.

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Trimeric Env spikes

Crystal structures of gp120 monomers have been available for over a decade [17], but the crystal structure of the trimeric Env spike is not available, though it has been modeled using monomer structures and cryo-electron tomography[63–65]. There are profound consequences to the oligomerization of the HIV-1 Env: trimer formation is a requirement for infectivity, results in the burial of neutralizing epitopes within oligomeric interfaces, and permits conformational epitope masking[66–68]. The QNEs that form upon trimerization of gp120 have been defined by various human and macaque monoclonal antibodies and are exceptionally potent[51–54], a fact that reflects a critical function of the QNEs that have not yet been defined.

3D visualization of and antigenic conservation within the variable loops

Recently, several 3D structural studies have provided significant insights into the conserved structural elements in the variable loops of gp120, suggesting windows of opportunity for designing immunogens expressing these conserved structures.

The V3 loop

Although the V3 loop is the least variable of the HIV-1 Env variable regions because, in part, it is the only one that is essentially constant in length, its sequence varies considerably across strains, and this variability is most pronounced in its "crown", ~14 amino acids in the middle of the V3 loop (Figure 1B). While the specificity of some V3 antibodies restricts their immunochemical and biologic function[69,70], some V3-specific monoclonal and polyclonal antibodies from infected individuals are able to neutralize diverse HIV-1 strains[19,36,43,71–74]. Furthermore, some individual V3-specific monoclonal antibodies can neutralize ~25–50% of Tier 1 and Tier 2 HIV-1 strains in which the correct epitope is present[19,37,43,72,75], polyclonal V3-specific responses to infection can neutralize Tier 1 and Tier 2 viruses from diverse clades [74], and vaccine-induced immune responses targeted to V3 can induce broader neutralizing activity than that seen in many human HIV^+ sera or in mixtures of human monoclonal antibodies[44].

Interestingly, crystallographic structures of the gp120 V3 loop crown from different virus strains in complex with various human monoclonal antibodies[41,59,76–79], and structures of the V3 loop freely arranged *in situ* on the core of gp120[80], all exhibit a V3 crown that forms variations of a common β-hairpin tertiary structure. Furthermore, the sequence variation of the V3 loop tends to cluster into a single continuous small zone when viewed in 3D space (Figure 1C and [40]). We hypothesize that the crown of the V3 loop might be organized into a folded (albeit highly flexible) globular domain, with the majority of its surface being structurally, and therefore antigenically, invariant. This structural perspective explains both the cross-reactivity of many V3-specific antibodies, which bind the conserved surfaces of V3, and the narrow specificity of some V3-specific monoclonal antibodies, which bind the small sequence-variable zone (Figure 1C and [40,41]). The discovery of conserved 3D structures in the sequence-variable V3 loop is consistent with the function of the V3 loop as a chemokine receptor-binding element: the receptor-binding surface of gp120 must be conserved to preserve virus infectivity, and these constraints apparently involve more of the V3 loop amino acid positions than they leave free to vary. This perspective of the V3 loop indicates which regions of V3 should be targeted with immunogens to elicit broadly neutralizing antibodies; it also reveals the region of V3 that induces only a strain-specific antibody response.

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The V1/V2 double loop

V2 is variable in both sequence and length (Figures 2 **and** 3), and as a result is a profoundly more variable region than V3. V2 is immunogenic in ~20–45% of HIV-infected humans[13] in contrast to V3, which induces antibodies in essentially all infected individuals[42]. Several human V2-specific monoclonal antibodies have been generated that cross-react with gp120 molecules from diverse HIV-1 isolates, and V2-specific monoclonal antibodies have also been produced from cells of infected chimpanzees and immunized rats[81–83]; this indicates that V2 might also contain some structurally conserved elements. Interestingly, many V2-specific monoclonal antibodies interact with the same amino acid residues or with overlapping regions of V2 (Figure 2C and [13,52,81,83,84]). Published studies document the neutralizing activity of human and chimp V2-specific monoclonal antibodies. Although many of these antibodies have little or weak neutralizing activity, some are quite potent[83,85,86].

Little is known about the 3D structure of the V2 loop, but the biologic advantage conferred by the integrin α4β7 binding motif in V2 [45] may constrain at least part of the V2 structure. This receptor-binding motif occurs in a region of V2 containing alternating and periodic conserved charged and hydrophobic amino acid residues typical of a folded domain. In addition, V2 contains a well-conserved disulphide bond common in small, loosely folded domains with high sequence variation in their 3D structural folds (such as the thioredoxin fold found in many proteins with known 3D structure)[87]. It is also notable that the C-terminal portion of the stem of the V2 loop is located directly adjacent to the CD4bs in crystal structures of gp120 liganded to CD4[17,80], and that some data suggest that the presence or absence of V2 affects recognition and neutralization by the anti-CD4bs antibody IgG1b12[66,88]. Further structural information is derived from a comparison of the length distributions of V1 and V2 (Figures 3A and 3B). The normal distribution exhibited by V1 is typical of random sampling from multiple optional structures, and therefore suggests that V1 is likely to be a dynamic, unfolded and disordered flexible loop that flickers between many conformations. The only other structural clues available for V1 come from the presence in the mid-region sequence observed in the most common length of repetitive glycosylation motifs (NXT/S shown as V1 residues 6–14, see Figure 2A).

The differing length distributions in V1 and V2 reflect biological functions that suggest 3D structural order within V2, but, as noted, structural disorder within V1. Specifically, the V2 length distribution exhibits a sharp drop-off in length from the most frequent length to shorter lengths, but a more gradual drop-off as the length increases, suggesting a viral mechanism that affords very little tolerance of V2 lengths shorter than ~40 amino acids. This pattern of length variation is typical of either a folded globular domain, which requires a critical length below which the fold cannot assemble, or inter-monomer contacts, which require a critical length below which the loop cannot reach over from one monomer to another. In both cases, the observed length distribution requires some V2 structural order. The available structural data, together with the observed cross-reactive immunological patterns of V2 antibodies, suggest that a similar, but more challenging, approach to vaccine targeting, as envisioned for V3, might also be successful for V2, but that V1 is probably not a suitable vaccine target.

V2/V3 complexes in the envelope trimer

There is now evidence that V2 and V3 can, together, form complex epitopes comprised of contributions from both variable loops. These complex epitopes exist preferentially or exclusively in the trimeric form of the HIV Env spike, defining them as quaternary epitopes (Box 1). Antibodies specific for quaternary epitopes were first described in $SHIV_{89.6P}$-infected macaques; although they were difficult to characterize in polyclonal sera, their

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activity seemed to map to a discontinuous epitope formed by V2 and V3 present on the virus' Env spikes[89]. Similar antibodies were found in an HIV-infected chimpanzee[90,91]. Remarkably, these antibodies were extremely potent as neutralizing reagents. The first human QNE-specific monoclonal antibody to be described, 2909, is extremely potent (IC_{50} < 1.0 ng/ml) and targets both the V2 and V3 regions[51]. The epitope to which it binds is found only on the surface of virions and on *env*-transfected cells, and does not exist on the gp120 monomer or recombinant soluble Env trimers[52,68], which indicates that this epitope is formed only when gp120 monomers form trimers in lipid membranes. Monoclonal antibody 2909 is highly strain-specific although it tolerates extensive amino acid sequence changes in V3[52]. The strain-specificity is the result of a lysine, rather than the more common asparagine, at residue 160 in V2 (Figure 2C and position 3 in Figure 2B, and [52,54]). So, although it is narrow in its reactivity, this monoclonal antibody defined the first quaternary epitope in the HIV-1 Env and revealed both tolerance for extensive variation in V3 and specificity constraints imposed by a single position in V2.

Recently, several QNE-specific monoclonal antibodies were generated from macaques infected with $SHIV_{SF162P4}$ [54]. Similar to monoclonal antibody 2909, these antibodies have potent but strain-specific neutralizing activity, target V2 and V3, and are limited in breadth of reactivity by residues in V2. So, macaques and humans both make similar potent QNE-specific antibodies.

Notably, two clonally related human monoclonal antibodies (PG9 and PG16) have now been described that also target V2 and V3 and possess potent neutralizing activity (IC_{50} < 1.0 ng/ml for many strains)[53]. Both these monoclonal antibodies can be considered QNE-specific antibodies although PG9 reacts weakly with monomeric gp120 as well as with trimeric gp140 and seems to target mainly discontinuous residues in V2. Monoclonal antibody PG16, a true QNE antibody, recognizes only the trimeric form of gp120 and interacts with discontinuous residues found in V2 and the crown of V3. Unlike human monoclonal antibody 2909 and the macaque QNE-specific monoclonal antibodies, PG9 and PG16 have broad activity, neutralizing 73% and 79% of 162 diverse pseudoviruses, respectively. The crucial difference that distinguishes PG9 and PG16 from monoclonal antibody 2909, and accounts for their neutralization breadth, is their dependence on the asparagine-linked carbohydrate moiety present in most HIV-1 isolates at position 160 in V2 (Figure 2C and position 3 in Figure 2B). The neutralization breadth of PG9 and PG16 suggests that the epitopes they target are conserved and not masked in most strains of HIV-1.

The immunologic reactivity and neutralizing activity of the human QNE-specific monoclonal antibodies show that they can readily tolerate extensive amino acid changes in V3[52,53], and that broad or narrow activity is dependent on V2 variation. Furthermore, the broad neutralizing activity of PG9 and PG16 demonstrates that the quaternary epitopes recognized are composed of conserved elements in the V2 and V3 sequence-variable regions and are the among most accessible of all neutralizing epitopes present on the CD4-unliganded form of the trimeric Env spike. The modelled Env trimers[63–65], provide clues for the visualizing the 3D structures of V2/V3 contacts, which probably occur within the many possible unliganded forms of the trimer. The resolved 3D structure(s) of trimers in QNE bound conformations should ultimately be highly informative for vaccine design.

In summary, recent data reveal conserved targets for broadly reactive antibodies in the sequence-variable V2 and V3 loops of gp120 and on quaternary structures formed by these loops in the trimeric Env. These data explain the broad immunological and functional cross-reactivity of some of the monoclonal antibodies specific for these regions and provide a framework for conceptualizing their value in vaccine design.

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Epitope masking from a structural perspective

Although masking is often described in terms of the ability of glycans and other structural features to prevent or decrease the neutralizing activity of V3-specific antibodies[19,34,38,47,92], epitope masking is a phenomenon common to *all* HIV-1 neutralizing epitopes, and is the result of a multitude of factors[67]. For example, the 3D structure of a particular epitope can assemble by adopting the “correct” conformation that clusters the key antibody-binding amino acid side-chains together into a single structure, but the protein may be designed to easily “flicker” out of that conformation, which would be observed as masking of the epitope from its cognate antibody. Similarly, other protein loops or glycans might “flicker” in to cover an epitope, which would also be observed immunologically as partial or complete masking. The result is that, although antibodies may be elicited by immunization targeting epitopes broadly present in circulating viruses, the effective neutralization of these antibodies *in vivo* is reduced to an uncertain extent by different types of masking. Masking can sometimes be overcome when particular conformations are stabilized by various molecular ligands. For example, in some strains, V3 epitope accessibility is markedly increased by the binding of CD4 and certain gp120-specific monoclonal antibodies that induce and stabilize conformational changes in gp120[39,93,94]. The latter data suggest that exposure of “masked” epitopes may be accomplished, and effective neutralizing activity achieved, by inducing “unmasking” antibodies along with antibodies to the “masked” epitope(s). Yet another perspective on masking is provided by some monoclonal antibodies that have differential neutralizing activities despite the fact that they target similar regions of the Env (Figure 2C). Binding of V2-, V3-, and QNE-specific antibodies to overlapping regions of V2 and V3 has different effects which may depend on the molecular dynamics of V2 and V3 loops in different conformations of the trimer and/or on the kinetics of V2 and V3 movement as they flicker between low and high-energy configurations[95]. Masking may, therefore, reflect the *functional* as well as the *physical* characteristics of an epitope in any of a myriad of molecular conformations. Hypothetically, residues in V2 and V3 may form the epitope(s) recognized by QNE-specific antibodies only when V2 and V3 interact in a configuration that leads to virus infectivity. In contrast, individual V2- and V3-specific antibodies may only recognize the epitopes on their respective loops when these loops occupy different space, i.e., are not in contact with one another, as in trimeric spikes that are not in an infectious conformation. The epitopes in the latter case would appear to be “masked”, since the anti-V2 and anti-V3 antibodies may not neutralize,

The conundrum of masking may, consequently, yield to further studies that can only be addressed by interdisciplinary studies that include structural as well as immunological and virological elements.

Conclusions and the way forward

Although the HIV-1 Env glycoproteins use variation, both in terms of sequence and conformation, to evade neutralization, there are nevertheless regions of structural conservation that can be targeted for vaccine design. This is supported by data showing that sequence-variable loops of HIV-1 gp120 can give rise to a spectrum of antibodies, some with very narrow specificities for only one or a few strains of HIV-1, and others with broad immunological and neutralizing activities against diverse HIV-1 strains. This is particularly true of antibodies specific for V2 and V3, and for the QNEs formed by these two regions of gp120. The immunological cross-reactivity of many of the monoclonal antibodies specific for these epitopes can now be explained by the structural conservation of 3D shapes within these sequence-variable loops and in the context of the functional requirements that limit the quality and quantity of sequence variability. This synthesis of the structural, functional, and

antigenic conservation of sequence-variable regions of gp120 provides a paradigm for the rational design of recombinant immunogens: it illuminates how form must follow function, it recalls how form relates to antigenicity, and it suggests how immunogens can be rationally designed to focus the immune response towards the portions of structures that elicit broadly reactive antibodies and away from those regions that elicit antibodies with narrow reactivity. Such rationally-designed immunogens will be modelled on the detailed 3D structures of epitopes recognized by families of cross-clade and broadly neutralizing antibodies, targeting conserved structural features and eliminating or replacing those elements that narrow the antibody specificity. New techniques have led to an explosion in the number and specificities of monoclonal antibodies that can be derived from patient specimens[19,20,51,53,73]. Other newly discovered epitopes and as yet unidentified epitopes recognized by serum antibodies with HIV-1 neutralizing activity[10,73,74,96–98] will provide additional targets beyond those that have been described here. Using structural and computational biology to rationally design recombinant epitope-scaffold immunogens based on neutralizing epitopes in the variable regions of Env has the potential of developing immunogens that elicit antibodies with greater specificity and potency than is currently achieved when various forms of gp120 and/or gp41 are used. Indeed, until recently, there has been limited success in inducing broadly reactive polyclonal neutralizing antibody responses that target selected epitopes in gp120 and gp41([6–9] and reviewed in [99]). However, focusing the immune response on neutralizing epitopes through the use of epitope-scaffold vaccines designed to present conserved structural elements and to eliminate those that narrow antibody specificity is beginning to yield modest successes[44]. These techniques hold the promise of constructing immunogens that elicit polyclonal antibody responses specific for particular chosen epitopes with finer specificity and greater potency than is currently achieved.

BOX 1 Types of Antibody-binding Epitopes

An antigenic determinant, also called an epitope, is a region of an immunogenic molecule that is recognized by the immune system, specifically by antibodies and T cell receptors.

Linear epitopes in proteins are composed of continuous stretches of amino acids derived from the protein sequence. Although antibodies that target “linear” epitopes can react with linear peptides, they might not necessarily react with contiguous stretches of amino acids within the peptides, but rather recognize discrete residues within the peptides that fold into particular conformations. This might result in greater affinity of the antibody for the native molecule compared with the relevant peptide[100]. Conformation contributes to most “linear” epitopes. Examples include the epitopes recognized by many gp120 V3-[41], C5-[101], and gp41-specific monoclonal antibodies[102,103] (Table 1).

Discontinuous epitopes are composed of amino acids that are in close proximity in the folded protein, but that are distant when unfolded. By definition, these epitopes require some, or extensive, secondary and/or tertiary protein structure. Hence, they are often referred to as “conformational” epitopes. Examples include the human recombinant antibody IgG1b12, which reacts with residues in several regions of gp120 [104], and human monoclonal antibody 17b, which reacts with the CD4-induced (CD4i) epitope[16,21,105] (Table 1). These can also be referred to as compound epitopes.

Quaternary epitopes are created by protein–protein interactions that occur upon multimerization. It is as a result of such reorganization that many proteins (such as enzymes and the trimeric gp120 spike of HIV-1) carry out their physiological function. An antibody that reacts with a true quaternary epitope will not interact with the individual monomeric subunits. Examples include monoclonal antibodies 2909 and PG16, which react with the trimeric form of gp120 on the surface of virions or *env*-transfected cells but not with monomeric gp120[51,53] (Table 1). However, some antibodies that react with

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quaternary epitopes react weakly with the monomeric subunits, but more strongly with the trimers. This seems to be true of monoclonal antibody PG9 [53]. Multimerization can result in changes in quaternary structure within individual subunits or through reorientation of the subunits relative to each other, so regions contributing to the epitope can be inter-molecular (*trans*) or intra-molecular (*cis*).

Acknowledgments

We are grateful to D. Almond and J. Swetnam for assistance in preparing the figures, and to C. Hioe, M. Totrov, and X.-P. Kong for contributing ideas to, and helping shape this article, which includes studies supported by grants from the Bill and Melinda Gates Foundation, the US National Institutes of Health (grants HL59725, AI36085, DP2 OD004631, and AI084119), and the Department of Veterans Affairs.

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Biographies

Susan. Zolla-Pazner received her Ph. D. from the University of California, San Francisco Medical Center in Microbiology. Her doctoral research on the immunochemical analysis of antibody specificity has formed the foundation of her work, leading to studies of immunoregulation of B cells, molecular mimicry among proteins, analyses of protein structure-function relationships, and the prevention, treatment and diagnosis of human infectious diseases. She participated in the description of the immunodeficiency of the first AIDS patients in 1981. Her current work focuses on the development of an HIV vaccine that will induce protective antibodies. She is currently Professor of Pathology at New York University School of Medicine and Director of AIDS Research at the New York Veterans Affairs Medical Center.

Timothy Cardozo received his M.D. from New York University School of Medicine, and his Ph.D. in Biochemistry from New York University. After medical residency training and postdoctoral work at New York University and work as a computational structural biology consultant for Molsoft, LLC (La Jolla, CA), he became an Assistant Professor at New York University in 2005. His research includes structure-based HIV immunogen design, novel informatics approaches, novel drug discovery approaches, clinical translational research in skin diseases, and protein design.

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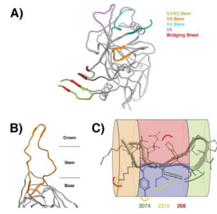

Figure 1.

A. Ribbon diagram of the crystallographic structure of the gp120 monomer bound to CD4. The locations on the structure are shown for the variable regions and bridging sheet (colored ribbons and color key). This gp120 monomer structure is adapted from Kwong et. al[17] and represents the truncated, deglycosylated conserved core of gp120 without the variable loops. The colored regions indicate the locations of the variable regions.

B. Ribbon diagram of the crystallographic structure of the V3 loop *in situ* on gp120 from the crystal structure solved by Huang et. al.[80] indicating the base, stem and crown regions of the V3 loop.

C. Zones of sequence variability mapped onto the β-hairpin conformation of the V3 crown (adapted from [40]). The crystallographic structure of the V3 crown is shown as a ribbon diagram, with commonly occurring side chains shown in stick depiction. A multi-colored illustrative cylinder is superimposed on the structure to highlight the four zones of the V3 crown[41]: the arch (green), the hydrophilic face (red) and hydrophobic face (blue) of the circlet, and the band (brown). Only the hydrophilic face has high sequence variability, but the amino acids that constitute it are distributed widely in the linear protein sequence, which obscures its existence unless the 3D structural context is appreciated. Side chains bound by neutralizing monoclonal antibodies are annotated with colored lines and a color key: monoclonal antibody 268 neutralizes only a few HIV strains and engages various side chains including one in the variable hydrophilic face of V3; monoclonal antibodies 3074 and 2219 neutralize viruses from several HIV-1 subtypes[37] and avoid engagement of the hydrophilic variable zone.

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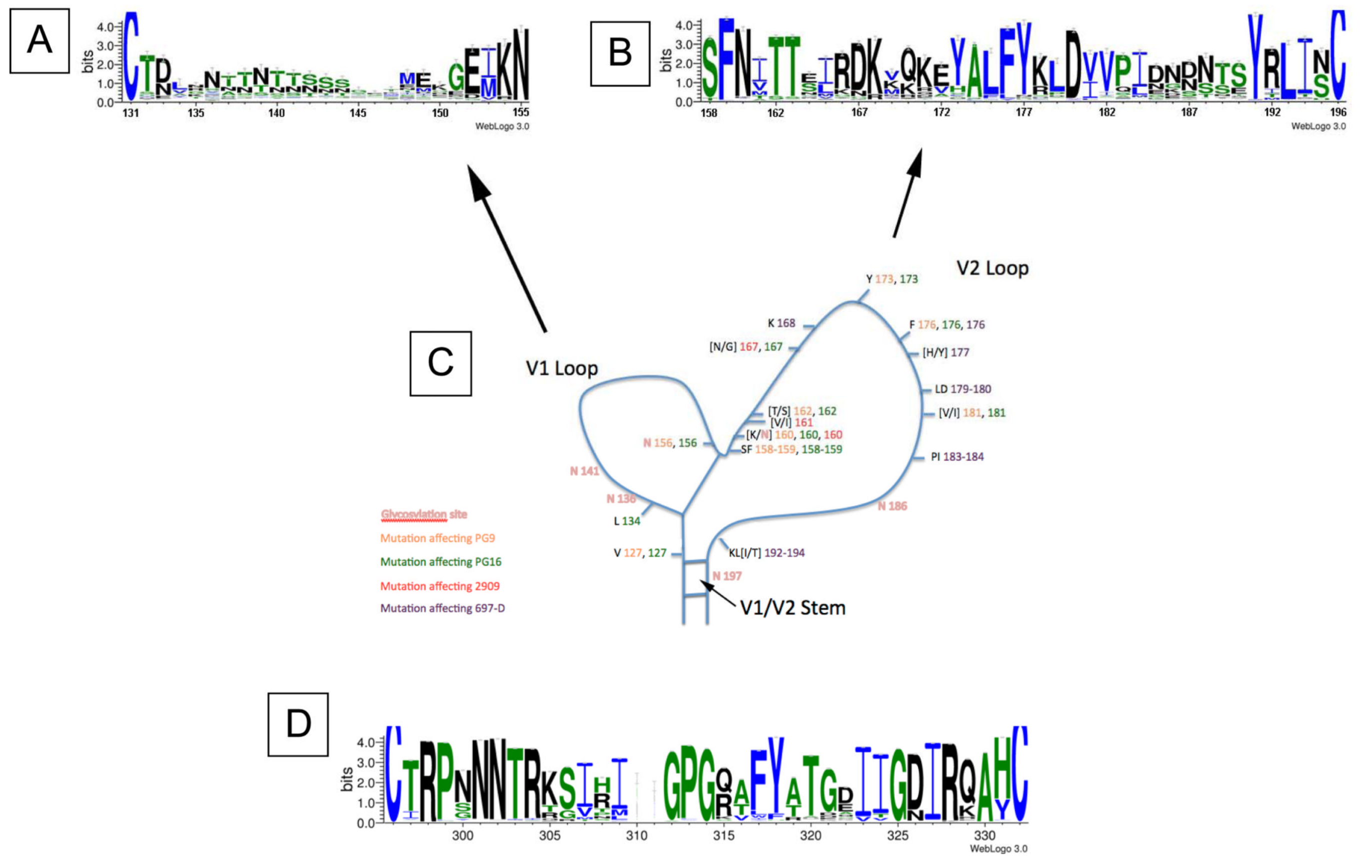

Figure 2.

Conserved and variable residues in the V1, V2, and V3 loops of gp120.

A. Sequence Logo depicting the amino acid conservation pattern across a multiple alignment of many V1 loops, each of which was selected because it has the most common V1 length depicted in Figure 3A. Data used to derive the Sequence Logo were derived from LANL, using one sequence per patient, and all HIV-1 subtypes were included. The height of the letter indicates the degree of conservation of the most common amino acid at that position. Amino acids are colored according to their chemical properties: polar amino acids (G,S,T,Y,C,Q,N) are green, basic amino acids (K,R,H) are blue, acidic amino acids (D,E) are red, and hydrophobic amino acids (A,V,L,I,P,W,F,M) are black. The few N- and C-terminal amino acids of the V1 loop show reasonable conservation, but most of the loop fades to small letters representative of no conservation except for a run of asparagines (N) and threonines (T) near the center suggesting a glycosylation region.

B. Sequence Logo depicting the amino acid conservation pattern across a multiple alignment of many V2 loops, each of which was selected from LANL (one sequence per patient, all subtypes included) because it has the most common V2 length depicted in Figure 3B. The height of the letter indicates the degree of conservation of that particular most common amino acid at that position. Although the V2 loop has many more insertions and deletions than the V3 loop, it has a degree of amino acid conservation approaching that of the V3 loop when one controls for loop length as explained above.

C. Amino acid positions and glycosylation sites that are implicated in the binding of V2-specific monoclonal antibody 697 and QNE-specific monoclonal antibodies PG9, PG16 and 2909 are mapped onto a schematic illustration of the V1/V2 loop structure. Map notations are colored according to the color key below the illustration. Brackets indicate more than one commonly occurring amino acid at a single position. The individual sites associated

with each single Ab are distributed throughout the V1/V2 loop linearly, but must cluster in 3D space into one or a few overlapping epitopes. The amino acid numbering in this panel may not exactly match that in Figrues 2A and 2B due to the variation in V1 and V2 lengths. **D**. Sequence Logo in the same fashion as Figures 2A and 2B depicting the amino acid conservation pattern across a multiple alignment of all V3 loops from LANL (one sequence per patient, all subtypes included)

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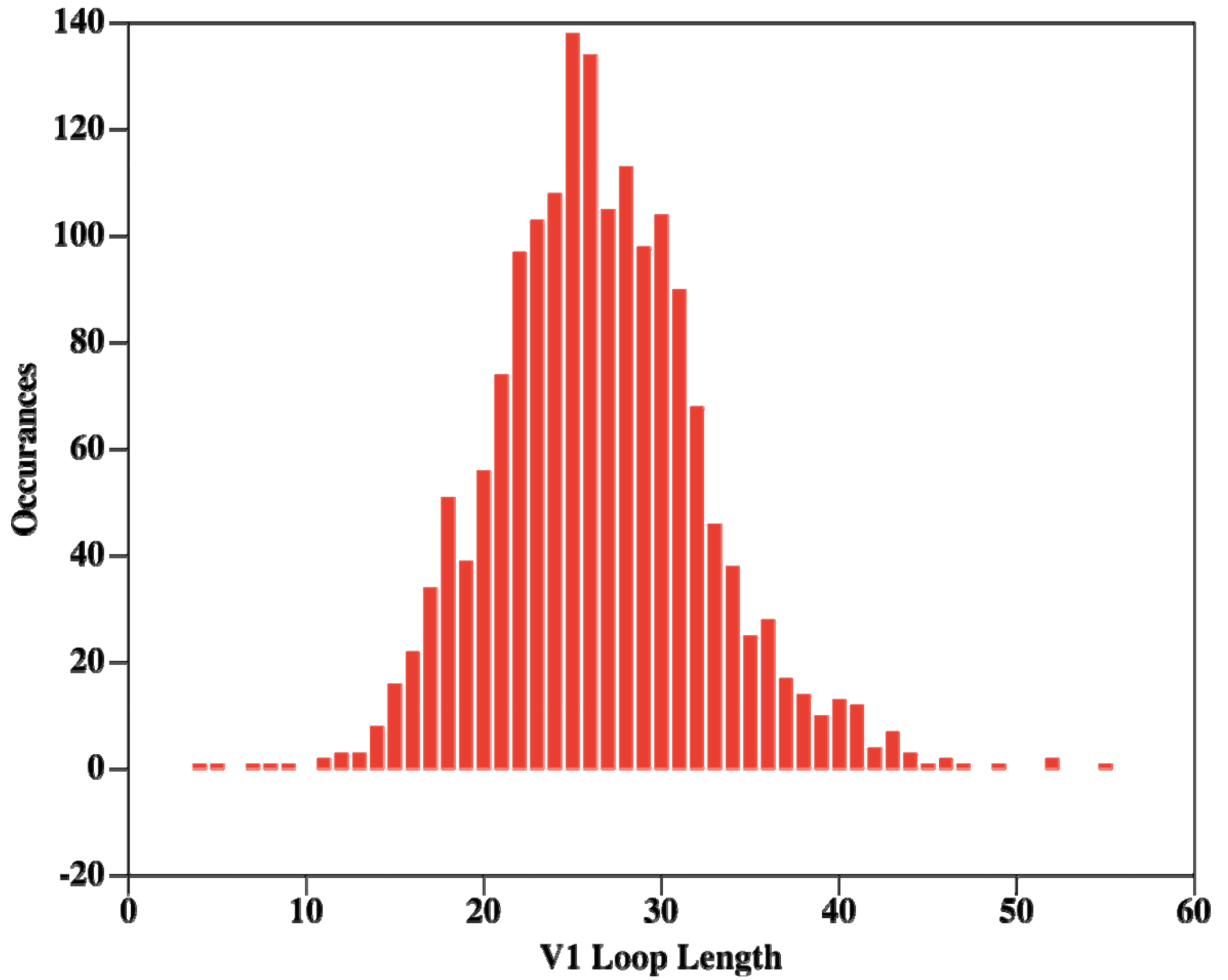
3A



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3B

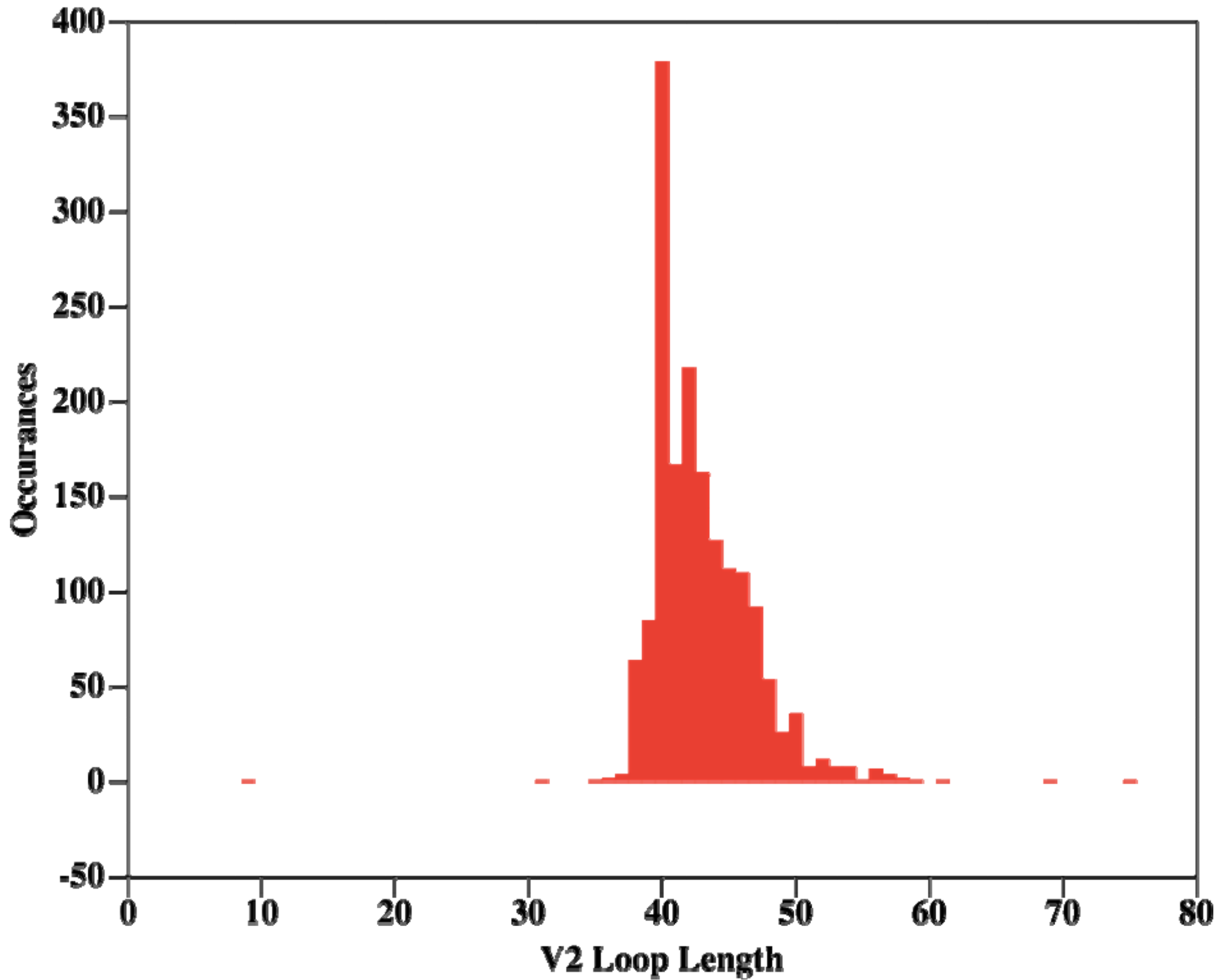

Figure 3.

Histograms showing the length distributions of the V1 **(A)** and V2 **(B)** loops from recorded HIV-1 sequences in LANL. The histograms shows the number (*y*-axis) of recorded viruses exhibiting each V1 or V2 length (number of amino acids, (*x*-axis) from zero to the maximal recorded length, so, for example, the origin shows that no viruses are recorded to have entirely deleted V1 or V2 loops (length of loop = 0).

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Table 1

Characteristics of human envelope-specific neutralizing monoclonal antibodies

Epitope	mAb	Epitope on	Region(s) Recognized	Epitope Type
CD4 binding site (CD4bs)	IgG1b12 HG16 VRC01 VRC03	gp120	C2, C3, C4, V5, C5	Discontinuous
CD4-induced (CD4i)	17b X5	gp120	Bridging sheet	Discontinuous
Complex Carbohydrate	2G12	gp120	Carbohydrate moieties in C2, C3, V4	Discontinuous
V3	447/52-D 2219 3074 HGN194	gp120	V3 loop	Linear (conformational)
Quaternary neutralizing epitopes (QNEs)	2909 PG9 PG16	gp120 trimer	V2/V3	Quaternary
Membrane proximal external region (MPER)	2F5 4E10 Z13	gp41	Protein & lipid	Linear (conformational)