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Minireview

Structure and Design of Broadly-Neutralizing Antibodies Against HIV

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Since the discovery more than 30 years ago of human immunodeficiency virus (HIV) as the causative agent of the deadly disease, acquired immune deficiency disease (AIDS), there have been no efficient vaccines against the virus. For the infection of the virus, the HIV surface glycoprotein gp120 first recognizes the CD4 receptor on the target helper T-cell, which initiates HIV fusion with the target cell and, if unchecked, leads to destruction of the patient's immune system. Despite the difficulty of developing appropriate immune responses in HIV-infected individuals, patient sera often contain antibodies that have broad neutralization activity, indicating the possibility of immunological treatment and prevention. Recently, through extensive structural studies of neutralizing antibodies of HIV in complex with gp120, the critical mechanisms of broad neutralization against HIV have been elucidated. Based on these discoveries, the structure-aided designs of antibodies and novel scaffolds were performed to create extremely potent neutralizing antibodies against HIV. These new discoveries and advances shed light on the road to development of efficient immunological therapies against AIDS.

INTRODUCTION

Human immunodeficiency virus (HIV) is the causative agent for the widespread acquired immune deficiency syndrome (AIDS) (Barre-Sinoussi et al., 1983; Gallo et al., 1984; Korber et al., 2000). HIV infects immune cells by recognizing the CD4 receptor on the surface of helper T-cells (Lane, 2010; Sattentau and Weiss, 1988). The recognition occurs by interaction of CD4 with the HIV surface glycoprotein gp120, which leads to shedding of gp120 from the membrane attached gp41 and the fusion of HIV with the target helper T cells by using the fusogenic peptide of gp41 (Harris et al., 2011; Wilen et al., 2012). The binding of CD4 to gp120 triggers a conformational change in the region and leads to opening of the co-receptor binding site that is originally hidden (Ju et al., 2012; Wilen et al., 2012).

Although viruses evade immune surveillance by varying amino acid residues of viral proteins, some amino acid residues must be invariant. Those critical residues include the viral surface protein residues involved in the receptor binding and the catalytically critical residues of viral enzymes (Swetnam et al., 2010). Thus, antibodies that recognize invariant residues on the viral surface can neutralize a wide range of virus isolates (Chi et al., 2007; Thali et al., 1992; Zhou et al., 2007). Although broadly neutralizing antibodies that target the V1/V2 and glycan have also been found in AIDS patients (McLellan et al., 2011), most broadly neutralizing antibodies recognize the conserved CD4 binding site (Chen et al., 2009; Zhou et al., 2007; 2010).

Despite the benefits of broadly neutralizing antibodies to the host, such antibodies are not efficiently generated in patients because the critical residues for CD4 binding are hidden by neighboring residues as well as by carbohydrate chains (Calarese et al., 2003; Haynes et al., 2005; Ofek et al., 2004; Sanders et al., 2002). Thus, intelligent approaches would be necessary to efficiently generate the broadly neutralizing antibodies. Recently, structures of several broadly neutralizing HIV antibodies in complex with gp120 were determined to show detailed mechanisms and critical interactions for efficient neutralization (Chen et al., 2009; McLellan et al., 2011; Zhou et al., 2007; 2010). The structural information on broadly neutralizing antibodies led to the design of extremely potent antibodies that mimic critical interactions of CD4 with gp120 (Diskin et al., 2011).

In addition to the structure-aided modification of specific motifs, protein design technologies were further advanced so that the design and incorporation of functional motifs into the convenient scaffolds became possible (Fleishman et al., 2011; Looger et al., 2003; Siegel et al., 2010). The scaffold design technology was successfully used in creating novel proteins grafted with the CD4 binding motifs of gp120 (Azoitei et al., 2011). We will review the recent discoveries on the atomic-level mechanisms of CD4 binding site-targeting broadly neutralizing antibodies as well as the design principles of potent neutralizing antibodies and vaccine candidates. The potency and breadth of newly designed neutralizing antibodies shed light on their therapeutic applications for treatment and prevention of AIDS.

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STRUCTURAL MECHANISM OF BROADLY NEUTRALIZING ANTIBODIES

Despite the difficulty of the immune system in developing effective strategies for diverse strains of viruses, it was found that patient sera often have antibodies with broadly neutralizing activities against some or most of HIV strains (Gonzalez et al., 2010). The broadly neutralizing antibodies with sufficient potency and breadth could be used in prophylactic vaccination to treat the viral diseases. If one designs proteins or peptides that could elicit broadly neutralizing antibodies, those materials would function as efficient preventive vaccines against highly variable viruses.

The anti-HIV antibody b12 (Burton et al., 1994) is one of only two broadly neutralizing antibodies, capable of neutralizing various primary isolates of HIV identified prior to recent studies that are the main subject of this review. Antibody b12 is a representative of CD4 binding site targeting (CD4BS-targeting) antibodies, which are also found for many anti-HIV antibodies that have a more restricted range of neutralization. The crystal structure determination of Fab b12 in complex with HIV gp120 revealed that the antibody interacts with the CD4 binding site of gp120 (Fig. 1), confirming the previous expectation that the invariant regions of viral surface protein are the target of broadly neutralizing antibodies (Zhou et al., 2007).

CD4 makes its initial contacts with the structurally invariant outer domain of gp120 that is on a constitutively exposed surface of the envelope spike (Cordonnier et al., 1989; Olshevsky et al., 1990). The nature of the CD4 interaction with gp120 was defined from structures of complexes of the D1D2 domains of CD4 with core constructs of monomeric gp120, showing essentially identical interactions for gp120 from a laboratory-adapted HIV strain (Kwong et al., 1998) and from a primary isolate (Kwong et al., 2000). Antibody b12 targets the same region of gp120 as for the interaction with CD4 (Zhou et al., 2007), binding primarily to the outer domain of gp120 whose conformation is well maintained in both CD4-bound and unbound structures of gp120 (Chen et al., 2005; Kwong et al., 1998; 2012). Although CD4 and b12 approach gp120 in a similar manner, the b12-bound conformations of gp120 inner domain and bridging sheet differ greatly from those of CD4-bound gp120 (Fig. 1) and the contact surfaces on gp120 differ for the two partners (Zhou et al., 2007). The energetics of interaction also differs with a greatly reduced entropic penalty for gp120 binding by b12 as compared to CD4, indicating residual flexibility in the b12-gp120 complex.

Unlike the broadly neutralizing antibody b12, most CD4BS-targeting antibodies do not neutralize HIV efficiently (Kwong et al., 2002; Starcich et al., 1986; Wyatt et al., 1998). The CD4BS-targeting antibodies F105 and b13 are two representative examples of those non-neutralizing CD4BS-targeting antibodies. Crystal structure determinations of gp120 in complex with F105 or b13 revealed delicate differences between neutralizing and non-neutralizing antibodies (Chen et al., 2009). In the structure of F105-gp120 complex, F105 mimics CD4 in many aspects (Chen et al., 2009). An important interaction of CD4-gp120 interaction is mediated between Arg59 of CD4 and Asp368 of gp120. The heavy chain residue Arg100F (the 6th residue of seven amino acid insertion in the position 100, PDB code: 3HI1) of F105 precisely mimics this interaction. Furthermore, the approach angle of F105 is similar to that of CD4, and the area of the CD4 binding region of gp120 occupied by F105 is 18% greater than that of b12.

Despite similarities in interactions and some advantages over the neutralizing antibody b12, one critical feature prevents F105 from being broadly neutralizing. In the F105-gp120 complex structure, the four-stranded bridging sheet of gp120 was significantly different from the CD4-bound state (Fig. 1). This conformational distortion uncovers a hydrophobic surface of gp120 for the F105 binding and affects the stem region of the V1/V2 loops, resulting in the shift of the V1/V2 tip up to 40 Å. These changes are not likely compatible with the trimeric state of gp120 in the viral spike. Modeling of the F105-bound gp120 into the electron micrograph (EM) structure of the trimeric gp120 (Liu et al., 2008) revealed major clashes between individual protomers (Chen et al., 2009).

The structure of another non-neutralizing antibody b13 in complex with gp120 showed that b13 also bound gp120 similarly to b12 (Chen et al., 2009). However, variable domains of two antibodies b13 and b12 showed about 17° rotation when their gp120-complex structures were superimposed by using gp120 regions. Although small, the rotation moved the b13 epitope and the bridging sheet strands significantly, resulting in more pronounced movement of the V1/V2 loop. Modeling of this ligand-induced alteration to the EM tomogram of gp120 trimers (Liu et al., 2008) also revealed serious clashes between protomors (Chen et al., 2009).

To find neutralizing antibodies with improved characteristics, the screening of patient sera was performed by using the modified gp120 whose surface was engineered to have a stabilized CD4-binding site (Wu et al., 2010; Zhou et al., 2007). Among the newly identified antibodies, the broadly neutralizing antibody VRC01 exhibited exceptional breadth of neutralization for more than 90% of all major circulating HIV-1 subtypes (Wu et al., 2010). The structure of VRC01 in complex with gp120 revealed the key mechanisms for the improved neutralization (Zhou et al., 2010).

Most strikingly, compared to complexes of other CD4BS-targeting antibodies with gp120, the conformation of gp120 in complex with VRC01 is very similar to its conformation as bound to CD4 (Fig. 1). Moreover, VRC01 mimics key interactions that CD4 makes with gp120; namely, the C″ strands of VRC01 V_H and CD4 D1 interact similarly with CD4-binding strand β15 of gp120, and both Arg71 of VRC01 V_H and Arg59 of CD4 are in doubly hydrogen-bonded charge pairs with Asp368 of gp120. The equivalent of CD4 Phe43 is missing, however. The interaction of VRC01 with gp120 was focused on the conformationally invariant outer domain region of gp120, which accounted for about 87% of the VRC01-gp120 contact (Zhou et al., 2010). The remaining 13% was made with flexible inner domain and bridging sheet, but with discontinuous characters so that these extra interactions were not critical to overall interaction forces for the VRC01-gp120 contact. In comparison, about 33% of the CD4-gp120 contact was made with inner-domain and bridging-sheet surfaces, and some of these interactions played critical roles in the CD4-gp120 complex formation. The VRC01-gp120 interaction covers 98% (1989 Å^2 in surface area) of an identified site of vulnerability on gp120, 50% more than the area covered in the CD4 interaction. The focus of VRC01 on this vulnerable site of initial CD4 attachment allows it to overcome the glycan and conformational masking that diminish the neutralization potency of most CD4BS-targeting antibodies.

Another characteristic of broadly neutralizing antibodies is an ability to recognize both CD4-bound and unbound conformations of gp120. When various CD4BS-targeting antibodies including F105, 17b, VRC01 and b12 were tested for affinity with different constructs of gp120, the non-neutralizing antibodies

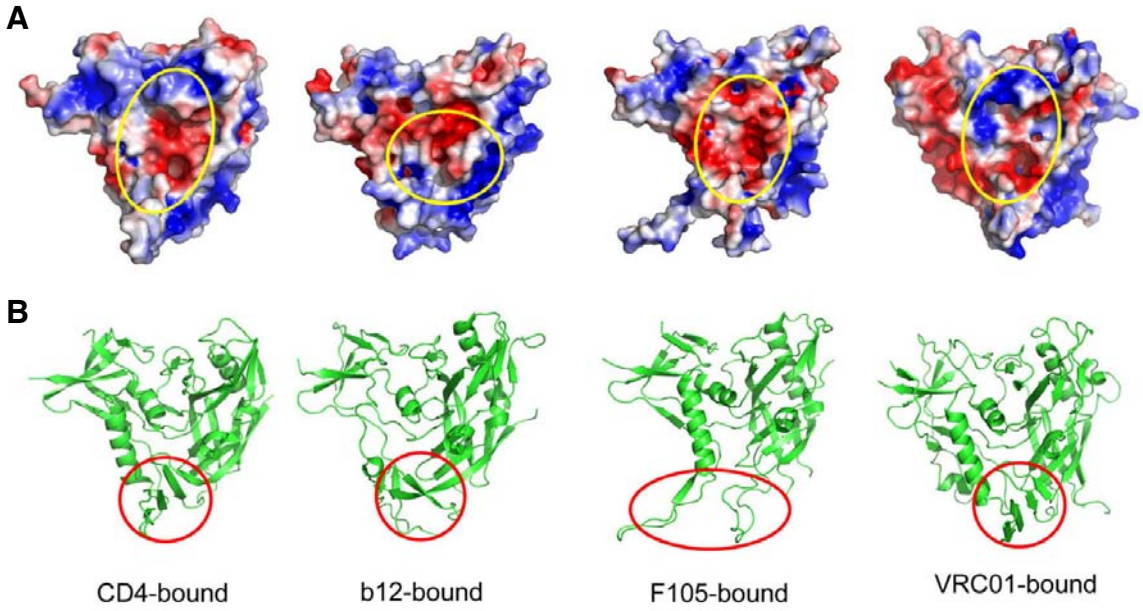

Fig. 1. Mechanism of broadly neutralizing antibodies. Structures of gp120 as bound to CD4 and CD4BS-targeting antibodies (b12, F105 and VRC01) are presented in the figure, showing only gp120 in each panel. (A) Electrostatic potential surface of gp120. Positive and negative potentials are shown in degrees of blue and red, respectively. CD4 interacting regions are indicated with yellow ellipses. (B) Ribbon drawings of the respective gp120 polypeptide backbones. Bridging-sheet segments are highlighted by red circles or ellipses. Among the CD4BS-targeting antibodies, F105 cannot neutralize HIV efficiently. Distortions of the bridging sheet of gp120 upon binding of F105 are incompatible with the trimeric gp120 state in the viral spike (see text). Antibody b12 is broadly neutralizing because it targets conserved outer-domain elements. Antibody VRC01 mimics CD4 quite faithfully, maintaining the same gp120 structure. PDB codes for the structures presented in the figure are 1GC1, 2NY7, 3HI1 and 3NGB for CD4, b12, F105 and VRC01 bound structures, respectively.

F105 and 17b and also soluble CD4 showed strong preference for one or the other of the CD4-bound or unbound conformations of gp120, whereas the broadly neutralizing antibodies VRC01 and b12 had high affinity to both conformations of gp120 (Zhou et al., 2010). This property of broadly neutralizing CD4BS-targeting antibodies appears to depend on their precise and focused interaction with the invariant CD4 binding region in the outer domain of gp120. Extra interactions with the inner domain and the bridging sheet such as shown in the non-neutralizing CD4BS-targeting antibodies can distort the bridging sheet and V1/V2 loop conformations leading to the incompatibility to the gp120 trimers in the viral spike.

STRUCTURE-BASED DESIGN OF POTENT ANTIBODIES

The antibodies recognizing the conserved CD4 binding site have different levels of neutralizing activity (Zhou et al., 2010). Because the efficiency of broadly neutralizing antibodies is dependent on the antibody's affinity for viral antigens and the breadth of neutralization, the affinity and neutralization breadth are two characteristics of HIV antibodies that have to be considered for generating efficient antibodies.

Broadly neutralizing antibody NIH45-46 is a clonal variant of the previously described antibody VRC01 (Scheid et al., 2011). NIH45-46 was isolated from a patient serum by using a YU2-gp140 trimer (Scheid et al., 2011), whereas VRC01 was from a screening of the same serum by using a resurfaced gp120 core (RSC3) as a bait (Wu et al., 2010). NIH45-46 was more potent than VRC01 in neutralizing various virus isolates and IC_{50} values of NIH45-46 for various HIV strains were 10-100 fold lower than those of VRC01 (Scheid et al., 2011).

The sequences of VRC01 and NIH45-46 can be aligned with high conservation levels in most regions of heavy chain and light chain variable domains including the complementarity determining region (CDR) loops. One exception occurs in the CDR3 loop of heavy chain variable domain (CDRH3) where the insertion of four residues named 99a-99b-99c-99d (Ala-Arg-Asp-Tyr) is observed in NIH45-46 (Scheid et al., 2011), resulting in a longer CDRH3.

Crystal structures of NIH45-46 alone and in complex with gp120 revealed that the antibody's elongated CDRH3 loop made extensive interactions with the CD4 binding region as well as the D and V5 loops of gp120 (Diskin et al., 2011). Three of the four inserted residues formed interactions similar to CD4-gp120 interactions, enhancing the interaction surface area considerably compared to that of VRC01. Importance of the four-residue insertion in NIH45-46 was verified by deleting the insertion from the antibody, which resulted in about 10-fold reduced neutralizing potencies (Diskin et al., 2011).

Contacts by the elongated CDRH3 loop render the total interaction surface of the NIH45-46-gp120 complex to more resemble that of the CD4-gp120 interaction than that of the VRC01-gp120 interaction. Other important interactions observed in the VRC01-gp120 complex including residues C-terminal to CDRH2 were also conserved in the NIH45-46-gp120. Interactions between the C" β-strand of CD4 domain D1 and the CD4-binding loop of gp120 also were conserved.

Although the four-residue insertion in NIH45-46 increased interaction surface with CD4 binding regions of gp120, the hydro-

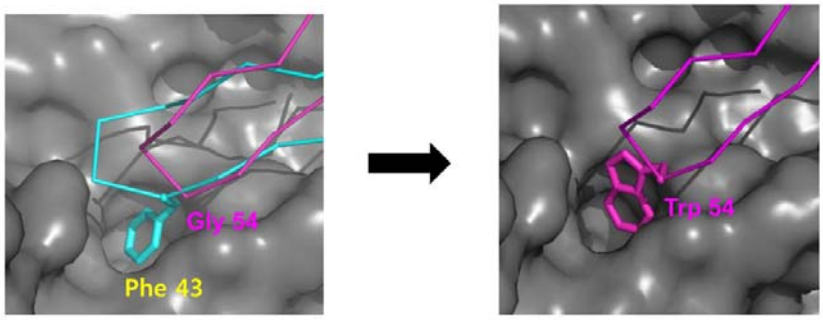

Fig. 2. Structure-based improvement of a broadly neutralizing antibody NIH45-46. The structures of CD4-gp120 complex and NIH45-46-gp120 complex structures (pdb codes 1GC1 and 3U7Y, respectively) were aligned by using the superposition of gp120 molecules in the two structures. In the left hand side figure, the gp120-interacting CD4 loop (cyan) and the corresponding NIH45-46 loop (magenta) were displayed on the surface representation (gray) of gp120 molecule. In the right hand side figure, Gly 54 of NIH45-46 was modeled to Trp 54 to show the fitting of the bulky side chain into the surface pocket of gp120.

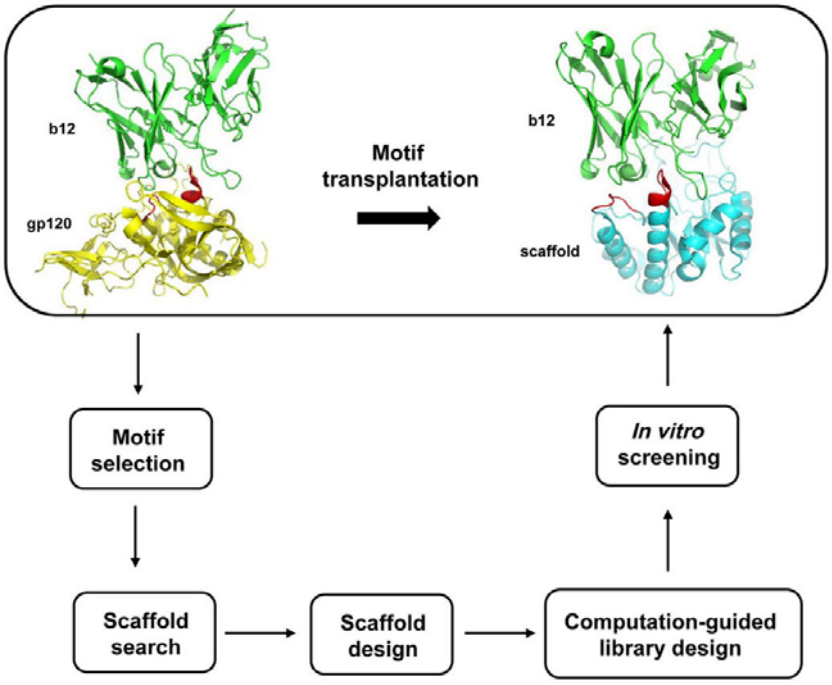

Fig. 3. The antigen grafting into a novel scaffold. The two major regions of gp120 (yellow) for the interaction with the broadly neutralizing antibody b12 (Fv region, green) were shown in red in the left hand side ribbon diagram. The helical loop and the extended loop of gp120 (red) represent the CD4 binding loop and the outer domain exit loop, respectively. In the right hand side ribbon diagram, the novel scaffold molecule (cyan) transplanted with the b12-interacting gp120 fragments (red) was shown in complex with b12 (green). The procedure leading to the antigen grafted scaffold generation was represented with boxes below the ribbon diagrams. PDB codes for the structures presented in the figure are 2NY7 and 3RU8 for gp120-bound b12 and scaffold-bound b12 structures, respectively.

phobic pocket for the binding of the bulky side chain of CD4 Phe43 was still not occupied by any of NIH45-46 residues. The interaction of CD4 Phe43 with gp120 is critical for the infection of HIV (Ryu et al., 1990; Wang et al., 1990) and the Phe43 interaction alone accounted for more than 23% of total interactions between CD4 and gp120, indicating that the interaction was crucial to the CD4-gp120 interaction during HIV infection (Kwong et al., 1998). The pocket also has been targets for small molecule development to inhibit CD4-gp120 interaction (Kwon et al., 2012; Madani et al., 2008; Xie et al., 2007).

In the structure of the NIH45-46-gp120 complex, the heavy chain residue Gly54 in NIH45-46 was located in the entrance of the CD4 Phe43 binding pocket of gp120 (Diskin et al., 2011). Gly54 was located in the CDRH2 loop that formed a beta-turn structure as in the Phe43-containing beta turn in the CD4 domain. The position of Gly54 was very close to Phe43 of CD4 with the Cα carbon distance of 1.4 Å (Fig. 2). To mimic the CD4-gp120 interaction, various mutations were created in the Gly54 position. Among the various substitutions, the G54W mutation exhibited potent neutralizing activities against different viral isolates, indicating that the substitution created favorable interactions with the CD4 Phe43 binding pocket (Fig. 2).

In particular, NIH45-46$_{G54W}$ efficiently neutralized the HIV strains that were not sensitive to the wild type NIH45-46. For some strains including T255-34 and 3718.v3.c11 strains that were poorly neutralized by NIH45-46, the increase in potencies by the G54W mutation was dramatic (more than 700-fold for the T255-34 strain and 2000-fold for the 3718.v3.c11 strain) (Diskin et al., 2011). The passive immunization has been shown to be effective in protecting people from HIV infection and becoming an important option for AIDS control (Hessell et al., 2007; 2009; Mascola et al., 2000). Thus, the improved potency and breadth of NIH45-46$_{G54W}$ suggests that the designed antibodies can be used for therapeutic purposes.

ANTIGEN GRAFTING INTO SCAFFOLDS

Designing novel proteins for desired functions can be performed in different levels from simple amino acid substitution to novel scaffold design (Baker, 2010; Correia et al., 2010; Looger

et al., 2003; Ofek et al., 2010; Siegel et al., 2010). Although the simple substitution of amino acids or loops in a parent molecule has been proven to be useful, the method can be applied only to improving functions of existing proteins. Recently, the grafting of desired residues and loops into an unrelated scaffold protein has shown successes in creating novel functional proteins (Siegel et al., 2010). In this approach, the search of appropriate scaffolds and the selection of trial designs for experimental validation are performed by extensive computation. In particular, the grafting of discontinuous functional motifs into an unrelated scaffold is challenging because it is not trivial to find matching scaffold that can accommodate motifs from different protein regions.

The discontinuous motif grafting procedure was applied to the design of novel proteins that mimicked the antigenic sites of HIV gp120 (Azoitei et al., 2011). From the crystal structure of the HIV gp120 in complex with the broadly neutralizing antibody b12, two regions of gp120 were identified to have extensive interactions with the antibody. The regions included the CD4 binding loop (residues 365-372) (Chen et al., 2005), and the outer domain exit loop (residues 472-476) (Zhou et al., 2007), which accounted for about 60% of the buried surface area in the complex of gp120 and CD4. These two segments also contributed about 80% of the gp120-CD4 binding energy (Azoitei et al., 2011).

The procedure of gp120 segment-grafting was performed in four stages: (i) scaffold search through entire PDB for accepting the gp120 segments; (ii) scaffold design for optimal connection of the segments and the scaffold; (iii) computation-guided mutagenesis library design for connecting segments; (iv) experimental *in vitro* screening for optimization of functional activity (Fig. 3). From the candidate scaffold-segments fusion designs, 62 variants were tested for expression in *Escherichia coli* and purification, which resulted in 25 soluble proteins (Azoitei et al., 2011). Among the 25 purified designs, one design (1bodx_03) with 39 mutations and 11 deletions from the parent protein bound to the neutralizing antibody b12 weakly but specifically exhibiting the dissociation constant (K_D) of 300 mM. The crystal structure of 1bodx_03 was determined to a high resolution (1.4 Å) to verify the design. However, although the scaffold structure was intact without major difference from the parent protein, the grafted loops did not appear in the electron density, indicating that the loops were flexible (Azoitei et al., 2011).

To optimize the design, the initial design (2bodx_03) was subjected to a whole-protein random mutagenesis library screening by using the yeast display method (Chao et al., 2006), which resulted in an improved construct (2bodx_R3) with two mutations (S117G and A118V) that had 10-fold higher affinity for b12 (K_D = 30mM). The affinity, however, was still several orders of magnitude lower than the b12-gp120 interaction of K_D = 20 nM (Zhou et al., 2007). Thus, a computation-guided library design approach was performed for the 21 positions that were judged to be important in the connection of segments, and optimal sequences were searched by using the yeast display screening. Through the yeast display library screening, 2bodx_42 with 17 mutations from the starting 2bodx_03 construct was found to have the b12-affinity of K_D = 166 nM which was >1,800-fold improvement over 2bodx_03. Introduction of another mutation from 2bodx_R3 further improved the affinity to 33 nM (2bodx_43). To see effects of the inclusion of more positions in the computation-guided library screening, seven more positions were added in the further optimization starting from 2bodx_43, which resulted in 2bodx_45 with a more improved affinity to b12 with K_D = 10 nM which was slightly better than the b12-gp120 affinity.

Crystal structure of the designed protein 2bodx_43 in complex with b12 was determined at 2.07 Å resolution (Azoitei et al., 2011) to verify the design principle. The structure exhibited a high degree of similarity with the b12-gp120 structure in the complex interface (Fig. 3). 2bodx_43 bound tightly only to b12 and the binding affinity for b13 was 10,000-fold less than that for b12, indicating the specificity of the designed protein. A similar antigen-grafting method was used in the design of novel proteins targeting influenza virus surface protein hemagglutinin where the hot spot residues were grafted into the shape complementary scaffolds, generating novel proteins with nanomolar affinity to the conserved region of hemagglutinin (Fleishman et al., 2011). Successes of the computer-aided antigen-grafting methods in both HIV gp120 and influenza virus hemagglutinin shed light on the development of vaccines that can generate neutralizing antibodies with sufficient potency and breadth.

CONCLUSIONS

The discovery of effective vaccines against AIDS has been hampered by HIV's highly variant surface antigen with flexible glycans and the conformational masking of the invariant CD4-binding region. From a series of structures of CD4 binding site antibodies in complex with gp120, the central mechanism of broad neutralization of HIV was revealed to be the precise recognition of invariant region of gp120 with strong affinity. The stray interactions over the boundary of invariant region by non-neutralizing CD4 binding site antibodies led to distortion of gp120 structures and incompatibility with the trimeric gp120 in the context of the viral spike. The structure-based improvement of a broadly neutralizing antibody by modifying CDRH2 loop to better recognize the CD4 Phe43-binding pocket of gp120 yielded the discovery of extremely potent and broadly neutralizing antibody which was expected to be useful in clinical settings. Presumably, continued affinity maturations could also arrive at similarly improved antibodies. Discovering vaccine strategies for effective elicitation of broadly neutralizing antibodies such as VRC01 and NIH45-46 presents a major challenge and an exciting opportunity.

Going beyond structural improvement at localized sites, the advanced protein design method involving the discontinuous antigen-loop grafting procedure shed light on a possible road to generating efficient vaccines. Aided by extensive computational and experimental searches for optimized structures, the antigen grafting into unrelated scaffolds was successful in creating novel proteins that can be used as vaccines to elicit broadly neutralizing antibodies *in vivo*. Although further refinement and clinical validation are needed, these advances seem to promise the discovery of efficient treatment and prevention methods against AIDS in the near future.

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