Published in final edited form as:

J Immunol Methods. 2012 February 28; 376(1-2): 150–155. doi:10.1016/j.jim.2011.12.008.

A strategy for phage display selection of functional domain-exchanged immunoglobulin scaffolds with high affinity for glycan targets

Alex Stewart, Yanyun Liu, and Jonathan R. Lai*

Department of Biochemistry, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461 USA

Abstract

Monoclonal antibodies are essential reagents for deciphering gene or protein function and have been a fruitful source of therapeutic and diagnostic agents. However, the use of anticarbohydrate antibodies to target glycans for these purposes has been less successful. Glycans contain less hydrophobic functionality than do proteins or nucleic acids, thus individual glycan– antibody interactions are relatively weak. Information encoded by glycans often involves subtle variations of branched oligosaccharides that cannot be detected with conventional antibodies. Here we describe a new phage display selection strategy for identification of high-affinity and specific glycan antibodies. We designed and characterized a phage clone that functionally displays the unique architectural scaffold of 2G12, an antibody that targets oligomannoses on the HIV-1 glycoprotein gp120. The two heavy chain variable domains of 2G12 exchange positions to create an extended recognition surface containing four oligomannose binding sites per IgG molecule. We designed and characterized a phage clone in which this domain exchange architecture was recapitulated as an antigen binding fragment dimer [(Fab)₂] on the phage surface by protein engineering. The functional display of the 2G12 (Fab)₂ fragment was validated by Western blot and phage enzyme-linked immunosorbent assay. Furthermore, we demonstrate that this 2G12 (Fab)₂ display system is amenable to selection of functional clones using a mock selection. These results provide proof-of-concept that the privileged 2G12 domain-exchanged scaffold can be used for design of novel antibody libraries that are biased toward glycan recognition.

Keywords

Antibody engineering; Phage display; Glycobiology

1. Introduction

Glycans (oligosaccharides) are critical information carriers in biology, yet progress toward understanding their roles has been hampered by lack of reagents that can detect subtle variations in glycan composition (Collins and Paulson, 2004; Prescher and Bertozzi, 2006). Antibodies and glycan-binding proteins (e.g., lectins) that recognize specific terminal sugars exist and are widely used, but these reagents have low affinity and are unable to distinguish among branched glycans. Subtle changes in the glycan composition at cellular surfaces, which can only be detected by discrimination of chemically similar high molecular weight branched oligosaccharides, are thought to signal major biological events and are associated with various disease states (Collins and Paulson, 2004; Prescher and Bertozzi, 2006).

Therefore, reagents that can distinguish branched oligosaccharides from one another would be of high value in glycobiology research. Furthermore, such reagents have great potential for diagnostic and therapeutic applications. Antibodies with these capabilities are difficult to obtain using hybridoma methods because glycans themselves tend to be poorly immunogenic, and it is difficult to target antibody response to regions that permit the desired level of discrimination among glycosylation patterns. Glycans have much less hydrophobic functionality than do proteins and nucleic acids; therefore, glycan–protein interactions tend to be lower affinity than protein–protein or protein–nucleic acid interactions (Collins and Paulson, 2004).

Recent progress in protein engineering has enabled identification of antibody fragments against various targets from de novo designed repertoires (Fellouse et al., 2007; Liu et al., 2011; Sidhu and Fellouse, 2006). In this approach, the diversity for such libraries is encoded by synthetic oligonucleotides ('synthetic antibodies'); the position and nature of the diversity elements are tailored to reflect amino acid compositions that have optimal physicochemical properties for antibody–antigen interactions (Fellouse et al., 2007). Therefore, the synthetic antibody approach circumvents the requirement for cloning variable domain segments from a natural immune repertoire. As a result, synthetic antibody libraries are not subject to biases of natural immune repertoires and the resulting antibodies can have enhanced properties. For example, synthetic antibodies with exquisite conformational or structural specificity have been isolated against several protein targets (Brawley et al., 2010; Gao et al., 2009). In addition, synthetic antibodies have been used to target post-translational modifications in high specificity (Newton et al., 2008), as well as nucleic acids, a class of antigens that has resisted traditional antibody isolation methods (Ye et al., 2008). The synthetic antibody approach is dependent upon a stable immunoglobulin framework that serves as a template for library design. The framework is chosen for desirable properties such as structural stability, tolerance to mutation, ease of expression, and predisposition toward particular antibody–antigen interactions. The scaffold of antibody 4D5, which appears highly biased toward protein–protein interactions, has served as the template for many protein-directed synthetic antibody libraries (Fellouse et al., 2007; Lee et al., 2004). Other frameworks that contain alternative CDR loop lengths and conformational propensities appear to have unique recognition properties (Da Silva et al., 2010; Shi et al., 2010). Specific scaffolds have been reported for use against peptide targets, which require a concave antigen binding site (Cobaugh et al., 2008), and single domain antibody scaffolds have also been described against several targets (Gilbreth et al., 2011; Wojcik et al., 2010).

The HIV-1 neutralizing antibody 2G12 is unique in its ability to target a complex glycan (the high molecular weight oligomannose residues on the envelope glycoprotein gp120) with high affinity (reported K_D of 5.6–16.1 nM for binding of gp120) and specificity (Calarese et al., 2003, 2005; Hoorelbeke et al., 2010). The structural basis for this selectivity arises from an unusual IgG1 architecture in which the two heavy chain variable domains on adjacent antigen binding fragments (Fabs) are domain-exchanged to form an extended and polyvalent glycan binding surface (Calarese et al., 2003). We surmised that this privileged scaffold could serve as a starting point for development of phage display synthetic antibodies biased toward glycan recognition. We report a selection strategy based on the 2G12 scaffold for identification of high-affinity glycan binding antibodies.

2. Materials and methods

2.1. Vector construction and mutagenesis

The bivalent Fab display phagemid pAS-Fab₂zip from Liu et al. (2011) was modified to generate bivalent 2G12 (Fab)₂ phagemid (p2G12-Fab₂zip). Splice overlap extension polymerase chain reaction was performed to generate DNA fragments containing the 2G12

light chain variable and constant domains (VL and CL, respectively) and the heavy chain variable and constant domains (VH and CH, respectively), which were subcloned into pAS-Fab₂zip. Kunkel mutagenesis was performed to generate mutants of p2G12-Fab₂zip (p2G12-Ala, p2G12-Gly, and p2G12-Arg, see Results and discussion) as previously described (Da Silva et al., 2010; Liu et al., 2011). Briefly, single-stranded, uridine-enriched DNA (ss-dU-DNA) of p2G12-Fab₂zip was prepared in *E. coli* CJ236 cells (New England Biolabs, NEB, Ipswich, MA) using standard protocols. Kunkel mutagenesis was performed using 5'-phosphorylated primers containing the desired mutations. Typical Kunkel mutagenesis reactions contained 3–5 µg of ss-dU-DNA, a three-fold excess of mutagenesis primer, three units of T7 DNA polymerase (NEB), and two units of T4 DNA ligase (Invitrogen, Carlsbad, CA). Reaction components were mixed and incubated at room temperature overnight and the products purified using a QIAgen (Valencia, CA) PCR purification kit. For the triple mutant p2G12-TriMut, a synthetic DNA fragment encoding the heavy chain fragment with mutations was obtained from a commercial supplier (Genewiz, South Plainfield, NJ) and subcloned as before.

2.2. Phage display

E. coli XL1-Blue (Stratagene Agilent Technologies, Santa Clara, CA) harboring p2G12-Fab₂zip were grown for ~4 h in 2xYT broth supplemented with 5 µg/mL tetracycline and 50 µg/mL carbenicillin at 37 °C. Helper phage M13-K07 (NEB) were added to ~10¹⁰ plaque-forming units (pfu)/mL, followed by 25 µg/mL kanamycin. The culture was grown for 16–18 h at 30 °C, the cells removed by centrifugation, and phage precipitated by addition of 3% (w/v) NaCl and 4% (w/v) PEG 8000. The phage were pelleted by centrifugation and resuspended in phosphate-buffered saline/0.05% (v/v) Tween 20 (PBS-T) containing 0.5% (w/v) BSA. Expression of an irrelevant single-chain variable fragment (scFv, pKZ52-scFv) was similar except the expression culture was grown at 37 °C.

2.3. Western blots

The p2G12-Fab₂zip phage (~10¹¹ infectious units (iu)/mL) were denatured by incubation at 100 °C for 10 min in SDS-PAGE buffer with or without β-mercaptoethanol. The solutions containing denatured phage particles were run on a 10% Tris–glycine HCl SDS-PAGE polyacrylamide gel and subsequently electrotransferred to polyvinylidene fluoride filter paper. Next, the filter paper was blocked with blocking buffer (Sigma, St. Louis, MO) for 10 min and then incubated with an anti-FLAG/horseradish peroxidase (HRP) conjugate (Sigma) in a 1/2500 dilution from stock in the blocking buffer for 1 h. The filter paper was washed five times with Tris-buffered saline containing 0.05% (v/v) Tween 20 (TBS-T) and developed with ECL development solution (Pierce/ThermoFisher Scientific, Rockville, IL). The blot was visualized on a GE ImageQuant LAS 4000.

2.4. Phage enzyme-linked immunosorbent assay (ELISA)

Wells of Costar EIA/RIA high-binding plates were coated with ~400–600 µg of antigen per well in phosphate-buffered saline (PBS) pH 7.0 at room temperature for 1 h. The well solutions were decanted, and unbound well sites were blocked with PBS-T/0.5% (w/v) BSA for 1 h. The wells were washed with PBS-T, phage solutions were added and allowed to bind for 30 min. The phage solutions were decanted, the wells washed 5–7 times with PBS-T. Next, a solution containing 1/2500 dilution of anti-M13/HRP conjugate (GE Healthcare, Piscataway, NJ) was added and allowed to bind for 30 min. The wells were washed with PBS-T ~5–7 times and developed using 3,3',5,5'-tetramethylbenzidine substrate (Sigma). The ELISA signal was quantified either by direct measurement of blue color absorbance (OD₆₅₀) or by quenching with sulfuric acid after 10–15 min and determining the OD at 450 nm.

2.5. Mock selections

Phage cultures containing mutations at selected contact residues at the HCDR1 and HCDR3 regions (p2G12-Ala, p2G12-Gly, p2G12-Arg) were spiked with small amounts of p2G12-Fab₂zip phage to produce input phage populations for the mock selection. These spiked input populations were subjected to a single round of mock selection. The protein target gp120 was immobilized in wells of Costar EIA/RIA as before. Solution containing the input phage population was added and allowed to bind for 1–2 h. Next, the wells were washed ~5–7 times with PBS-T. Treatment with 100 μ L of 100 mM glycine HCl pH 2.1 to each well for 10 min allowed for elution of phage. Subsequently, the solution was neutralized by addition to 50 μ L of 2 M Tris, pH 8. The populations were also subjected to a round of mock selection against control wells coated with BSA. The titers of the input and output populations were determined using standard methods. The frequency of WT (p2G12-Fab₂zip) to mutant (p2G12-Ala, p2G12-Gly, p2G12-Arg) in input and output populations was assessed by large-scale DNA sequencing.

3. Results and discussion

Antibody 2G12 is a unique immunological solution for high-affinity humoral response to carbohydrate cluster recognition (Calarese et al., 2003, 2005). This antibody binds high molecular weight oligomannose N-glycans on the HIV-1 envelope glycoprotein gp120 with nanomolar affinity (Calarese et al., 2003, 2005; Hoorelbeke et al., 2010); most other glycan antibodies bind with dissociation constants in the micromolar range. The unusually tight binding of 2G12 results from a unique domain exchange architecture that produces an extended antigen (glycan) interaction surface on the antibody (Calarese et al., 2003). Fig. 1A shows a schematic for the domain-exchange architecture of 2G12 — the only reported case in which such a dramatic adaptation of the IgG scaffold is afforded by somatic hypermutation and clonal selection. The crystal structure of the Fab fragment for 2G12, a dimer, is shown in Fig. 1B complexed with four Man₉GlcNAc₂ oligosaccharides (the glycan found on gp120) (Calarese et al., 2003). The basis for the domain exchange is rooted in a Ser→Pro mutation in the linker region between heavy chain variable and constant domains (VH and CH), which redirects the polypeptide chain in such a manner to promote the exchange of VH and VH' domains on adjacent arms of the IgG molecule (Fig. 1C) (Calarese et al., 2003). An extensive network of interactions among the VH and VH' domains provide additional stabilization for the domain exchange (Calarese et al., 2003). This unique architecture supports key structural features that endow 2G12 with its rare ability to recognize branched glycans in high affinity (Calarese et al., 2003). In 2G12, the domain VH–VH' domain exchange results in four glycan-binding pockets per IgG molecule. Two of these glycan binding pockets, the primary binding sites, are located at the VL–VH' and VL'–VH interfaces (Fig. 1A and B). These primary sites correspond to conventional antibody combining sites. Two secondary glycan-binding pockets, diametrically opposed, lie at the VH–VH' interface. This efficient and compact polyvalency, embedded within a single IgG molecule, form the basis for 2G12's ability to form high-affinity interactions with glycan moieties. Furthermore, due to an extended network of contacts with the glycan, 2G12 is able to discriminate Man₉GlcNAc₂ from other closely-related oligomannoses (Calarese et al., 2005). Our goal is to use this unique domain-exchange scaffold for the development of 2G12-based synthetic antibody libraries. We therefore sought to devise a method to functionally express the domain-exchanged architecture of 2G12 on the surface of M13 bacteriophage.

Most phagemid/helper phage display vector systems, in which Fab fragments are expressed as fusions to the minor coat protein pIII, result in low levels of display and uncontrolled number of Fab fragments. However, it is frequently thought this strategy yields mostly monovalently expressed clones. Since the domain-exchanged architecture of 2G12 is critical

to its high-affinity glycan binding, we required a system that would ensure the display of two Fab fragments in close proximity to allow the VH–VH' exchange. Several systems have been reported for the expression of (Fab)₂ dimer on phage; we and others have previously used phagemid vectors containing the IgG hinge region and GCN4 leucine zipper for this purpose (Lee et al., 2004; Liu et al., 2011). We postulated that a (Fab)₂-display construct bearing the 2G12 Fab sequence, and the IgG hinge segment and GCN4 downstream of the CH domain would allow the VH–VH' exchange since the GCN4 would promote dimerization during phage assembly, and the hinge region would stabilize this dimeric conformation by formation of interchain disulfide crosslinks. The design of this vector (p2G12-Fab₂zip) is shown in Fig. 2, and details of the design are described in the figure caption.

To determine if the 2G12 (Fab)₂ fragment was efficiently expressed, we prepared phage particles from p2G12-(Fab)₂zip using standard procedures and subjected these phage particles to Western blot analysis using a FLAG epitope on the light chain for detection (Fig. 3A). We observed a band corresponding ~150 kDa assembly under non-reducing conditions, the expected molecular weight of the dimeric 2G12 Fab-hinge-GCN4-(pIII-CT) assembly. When the phage were subjected to reducing conditions, a band of ~25 kDa was observed, which corresponds to the unassembled light chain. These results indicate that the bivalent Fab assembly is expressed on the surface of phage. We next performed phage ELISA to determine if the 2G12 (Fab)₂ fragment was functional. A strong ELISA signal for binding of phage particles produced from p2G12-(Fab)₂zip to wells coated with gp120 was observed but not for wells coated with BSA, and this gp120 binding signal was phage titer-dependent (Fig. 3B). Importantly, a control phage expressing the scFv of an irrelevant Ebola virus antibody (KZ52, pKZ52-scFv) did not exhibit binding to gp120. However, pKZ52-scFv did bind its native antigen (GP1/2) in a phage-dependent manner, indicating that the KZ52 scFv is functionally expressed. These results indicate that the binding signal of p2G12-(Fab)₂zip is specific for both the antibody and the native glycan antigen. Furthermore, the p2G12-(Fab)₂zip phage particles exhibited similar phage-dependent binding to wells coated with anti-idiotypic antibody M1G1, which binds to 2G12 in a manner that is dependent on proper assembly of the 2G12 heavy and light chains (Roux et al., 2004). These data suggest that the 2G12(Fab)₂ fragment is functionally displayed.

We created several site-specific mutants of p2G12-Fab₂zip to further explore the properties of our phage display strategy. Several residues have previously identified three residues at the VH–VH' interface that are critical for stabilizing the domain exchange (Calarese et al., 2003). We prepared a triple mutant of p2G12-Fab₂zip in which three of these positions were altered to alanine (I19A/F77A/Y79A, p2G12-TriMut). In addition, the crystal structure of the 2G12 Fab in complex with oligomannose revealed several positions in HCDR1 and HCDR3 that participate in antigen-binding contacts (Calarese et al., 2003). We created three additional variants in which a total of nine positions in these regions (A31, H32, T33, K95, L100, S100a, D1100b, N100c, and D100d) were altered from WT to Ala (p2G12-Ala), Gly (p2G12-Gly), or Arg (p2G12-Arg). These mutations are expected to be deleterious to glycan binding. We compared the binding of each of these clones to immobilized gp120 by phage ELISA (Fig. 3D). Since the mutants may exhibit differential display properties relative to WT, we also performed a phage ELISA against anti-FLAG antibody M2 that would allow evaluation of the (Fab)₂ expression. The functional ELISA (gp120) was performed at phage titers in which we observed similar signals for binding to antibody M2 (as indicated by the relative ELISA signal against M2 in Fig. 3D). This analysis should provide a direct comparison of the binding capacity for the expressed fragments irrespective of any differences in display. We found that the WT 2G12 clone (p2G12-Fab₂zip) had with significantly higher binding signal than did the domain interface triple mutant (p2G12-TriMut), or clones in which the contact HCDR1 and HCDR3 contact residues had been

altered (p2G12-Ala, p2G12-Gly, and p2G12-Arg). These results further indicate that the 2G12 domain exchange is functional.

Finally, to test the capacity of the p2G12-Fab₂zip system for selection of functional selection, we prepared mixtures of phage populations containing p2G12-Ala, p2G12-Gly, or p2G12-Arg spiked with p2G12-Fab₂zip. In theory, if the bivalent p2G12-Fab₂zip were amenable to selection of high-affinity clones, then the amount of the WT 2G12 clone (p2G12-Fab₂zip) should be enriched relative to the less functional mutant clones after selection against gp120. Various mixtures of p2G12-Ala, p2G12-Gly, or p2G12-Arg and p2G12-Fab₂zip were prepared (Table 1) and subjected to selection against immobilized gp120. Enrichment, defined as increased output phage titer from wells coated with gp120 relative to wells coated with BSA, was observed in all cases. The input and output populations for selection against gp120 were subjected to large-scale sequencing to determine the frequency of the WT 2G12 clone (p2G12-Fab₂zip); ninety-four random clones were sequenced for each of the populations. The frequency of p2G12-Fab₂zip increased dramatically after selection in all cases. These results further demonstrate that the p2G12-Fab₂zip display scaffold will be amenable to selection of functional domain exchanged scaffolds from a background of non-functional clones.

4. Conclusions

We report here a novel strategy for phage display selection of functional, domain-exchanged immunoglobulin scaffolds with high affinity for glycans. While (Fab)₂ fragments have previously been expressed on the surface of M13 bacteriophage (Fellouse et al., 2007; Lee et al., 2004; Liu et al., 2011), this work represents the first case (to our knowledge) in which the domain-exchanged scaffold of 2G12 has been functionally displayed. Protein engineering of the (Fab)₂ construct to include the GCN4 and IgG hinge regions downstream of the CH domain were critical to this strategy. This approach was sufficient to mimic the 2G12 architecture in the absence of the F_C region. Mutational analysis demonstrated that binding of the p2G12-(Fab)₂zip phage to gp120 is dependent on this domain-exchange, and on contact residues in HCDR1 and HCDR3. The 'privileged' 2G12 scaffold will serve as the starting point for development of synthetic antibody libraries that are biased for glycan recognition.

Acknowledgments

This work was funded by the Albert Einstein College of Medicine, and the NIH/NCI Innovative Molecular Analysis Technologies Program (R21-CA155472). A.S. was supported in part by a fellowship from the UNCF/Merck Science Initiative. We thank Lai-Xi Wang of the University of Maryland Institute of Human Virology for generously providing the gp120, and Erica Ollman Saphire of the Scripps Research Institute for providing the Ebola virus GP1/2.

Abbreviations

Fab	antigen binding fragment
CDR	complementarity determining region
VL	light chain variable domain
CL	light chain constant domain
VH	heavy chain variable domain
CH	heavy chain constant domain
ss-dU-DNA	single-stranded uridine-enriched DNA

PBS-T	phosphate-buffered saline containing 0.05% (v/v) Tween 20
scFv	single-chain variable fragment
iu	infectious units
HRP	horseradish peroxidase
TBS-T	Tris-buffered saline containing 0.05% (v/v) Tween 20
ELISA	enzyme-linked immunosorbent assay
ORF	open reading frame
RBS	ribosome binding site

References

- Brawley CM, Uysal S, Kossiakoff AA, Rock RS. Characterization of engineered actin binding proteins that control filament assembly and structure. *PLoS One*. 2010; 12:e13960. [PubMed: 21103060]
- Calarese DA, Scanlan CN, Zwick MB, Deechongkit S, Mimura Y, Kunert R, Zhu P, Wormald MR, Stanfield RL, Roux KH, Kelly JW, Rudd PM, Dwek RA, Katinger H, Burton DR, Wilson IA. Antibody domain exchange is an immunological solution to carbohydrate cluster recognition. *Science*. 2003; 300:2065. [PubMed: 12829775]
- Calarese DA, Lee HK, Huang CY, Best MD, Astronomo RD, Stanfield RL, Katinger H, Burton DR, Wong CH, Wilson IA. Dissection of the carbohydrate specificity of the broadly neutralizing anti-HIV-1 antibody 2G12. *Proc Natl Acad Sci U S A*. 2005; 102:13372. [PubMed: 16174734]
- Cobaugh CW, Almagro JC, Pogson M, Iverson B, Georgiou G. Synthetic antibody libraries focused towards peptide ligands. *J Mol Biol*. 2008; 378:622. [PubMed: 18384812]
- Collins BE, Paulson JC. Cell surface biology mediated by low affinity multivalent protein–glycan interactions. *Curr Opin Chem Biol*. 2004; 8:617. [PubMed: 15556405]
- Da Silva GF, Harrison JS, Lai JR. Contribution of light chain residues to high affinity binding in an HIV-1 antibody explored by combinatorial scanning mutagenesis. *Biochemistry*. 2010; 49:5464. [PubMed: 20518570]
- Fellouse FA, Esaki K, Birtalan S, Raptis D, Cancasci VJ, Koide A, Jhurani P, Vasser M, Wiesmann C, Kossiakoff AA, Koide S, Sidhu SS. High-throughput generation of synthetic antibodies from highly functional minimalist phage-displayed libraries. *J Mol Biol*. 2007; 373:924. [PubMed: 17825836]
- Gao J, Sidhu SS, Wells JA. Two-state selection of conformation-specific antibodies. *Proc Natl Acad Sci U S A*. 2009; 106:3071. [PubMed: 19208804]
- Gilbreth RN, Truong K, Madu I, Koide A, Wojcik JB, Li NS, Piccirilli JA, Chen Y, Koide S. Isoform-specific monobody inhibitors of small ubiquitin-related modifiers engineered using structure-guided library design. *Proc Natl Acad Sci U S A*. 2011; 108:7751. [PubMed: 21518904]
- Hoorelbeke B, Huskens D, Féris G, François KO, Takahashi A, Van Laethem K, Schols D, Tanaka H, Balzarini J. Actinohivin, a broadly neutralizing prokaryotic lectin, inhibits HIV-1 infection by specifically targeting high-mannose-type glycans on the gp120 envelope. *Antimicrob Agents Chemother*. 2010; 54:3287. [PubMed: 20498311]
- Lee CV, Sidhu SS, Fuh G. Bivalent antibody phage display mimics natural immunoglobulin. *J Immunol Methods*. 2004; 284:119. [PubMed: 14736422]
- Liu Y, Regula LK, Stewart A, Lai JR. Synthetic Fab fragments that bind the HIV-1 gp41 heptad repeat regions. *Biochem Biophys Res Commun*. 2011; 413:611. [PubMed: 21925149]
- Newton K, Matsumoto ML, Wertz IE, Kirkpatrick DS, Lill JR, Tan J, Dugger D, Gordon N, Sidhu SS, Fellouse FA, Komuves L, French DM, Ferrando RE, Lam C, Compaa D, Yu C, Bosanac I, Hymowitz SG, Kelley RF, Dixit VM. Ubiquitin chain editing revealed by polyubiquitin linkage-specific antibodies. *Cell*. 2008; 134:668. [PubMed: 18724939]
- Prescher JA, Bertozzi CR. Chemical technologies for probing glycans. *Cell*. 2006; 126:851. [PubMed: 16959565]

- Roux KH, Zhu P, Seavy M, Katinger H, Kunert R, Seamon V. Electron microscopic and immunochemical analysis of the broadly neutralizing HIV-1-specific, anti-carbohydrate antibody, 2G12. *Mol Immunol.* 2004; 41:1001. [PubMed: 15302162]
- Shi L, Wheeler JC, Sweet RW, Lu J, Luo J, Tornetta M, Whitaker B, Reddy R, Brittingham R, Borozdina L, Chen Q, Amegadzie B, Knight DM, Almagro JC, Tsui P. De novo selection of high-affinity antibodies from synthetic fab libraries displayed on phage as pIX fusion proteins. *J Mol Biol.* 2010; 397:385. [PubMed: 20114051]
- Sidhu SS, Fellouse FA. Synthetic therapeutic antibodies. *Nat Chem Biol.* 2006; 2:682. [PubMed: 17108986]
- Wojcik J, Hantschel O, Grebien F, Kaupe I, Bennett KL, Barkinge J, Jones RB, Koide A, Superti-Furga G, Koide S. A potent and highly specific FN3 monobody inhibitor of the Abl SH2 domain. *Nat Struct Mol Biol.* 2010; 17:519. [PubMed: 20357770]
- Ye JD, Tereshko V, Frederiksen JK, Koide A, Fellouse FA, Sidhu SS, Koide S, Kossiakoff AA, Piccirilli JA. Synthetic antibodies for specific recognition and crystallization of structured RNA. *Proc Natl Acad Sci U S A.* 2008; 105:82. [PubMed: 18162543]

Fig. 1.

Unusual domain-exchange architecture of 2G12. (A) Schematic of domain architecture for canonical IgG molecules (top) and 2G12 (bottom). Canonical IgG architecture results in two antigen binding sites at the interface of light and heavy chain variable domains (VL–VH and VL'–VH'). The VH–VH' domain exchange in 2G12 creates an extended antigen binding surface that contains two additional binding sites at the VH–VH' interface. (B) Crystal structure of the 2G12 Fab dimer in complex with Man₉GlcNAc₂ (orange) reported by Calarese et al. (2003). Domain colors as shown in panel A. (C) Fab monomer of 2G12 showing features that stabilize the domain exchange.

Fig. 2.

Design of the p2G12-Fab₂zip phagemid. The region upstream of the M13 minor coat protein pIII C-terminal domain (pIII-CT) contains two open reading frames (ORFs): one that encodes the light chain (VL-CL, an N-terminal FLAG sequence is included for detection); and a second that encodes the VH, CH, the IgG hinge region, a GCN4 leucine zipper linked to pIII-CT. Both ORFs are under the control of a single promoter; the intragenic region contains a ribosome binding site (RBS). The leader sequences OmpA and PelB direct the secretion of the light and heavy chains into the *E. coli* periplasm during phage assembly. The GCN4 segment is predicted to drive dimerization, and the bivalent assembly is stabilized by disulfide crosslinks in the hinge domain. The obligate dimer imposed by the GCN4 and hinge regions permits the functional VH-VH' domain exchange, which is critical for activity.

Fig. 3.

Functional expression of the 2G12 (Fab)₂ fragment on phage. (A) Western blot analysis of p2G12-Fab₂zip phage particles under reducing (r) and non-reducing (nr) conditions using an anti-FLAG/HRP probe (a FLAG epitope is included on the light chain for detection). The asterisk indicates a polyreactive phage protein. (B) Phage ELISA of p2G12-Fab₂zip or pKZ52-scFv phage against various targets at three phage titers (infectious units (iu)/mL). (C) Comparison of function among p2G12-Fab₂zip and mutants. The ELISA binding signal is normalized relative to the WT (p2G12-Fab₂zip) signal for both gp120 and anti-FLAG antibody M2.

Table 1

Sequence analysis from mock selection.

Input phage mixture	Input titer (iu/mL)	Output titer (gp120; iu/mL)	Output titer (BSA; iu/mL)	Frequency of WT in input population ^a	Frequency of WT in output population (gp120) ^a
p2G12-Fab ₂ zip/p2G12-Ala	6×10 ⁹	2×10 ⁶	1×10 ⁵	27/94 (29%)	62/94 (66%)
	3×10 ¹²	8×10 ⁷	5×10 ⁷	2/94 (2%)	14/94 (15%)
p2G12-Fab ₂ zip/p2G12-Gly	2×10 ¹⁰	2×10 ⁶	2×10 ⁵	3/94 (3%)	20/94 (21%)
p2G12-Fab ₂ zip/p2G12-Arg	4×10 ¹⁰	2×10 ⁶	1×10 ⁵	0/94 (0%)	10/94 (11%)

^aFrequency of the WT 2G12 clone (p2G12-Fab2zip) in the population was assessed by large-scale sequencing. In all cases, 94 clones were randomly sequenced.