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Anti-Idiotypic Monobodies Derived from a Fibronectin Scaffold

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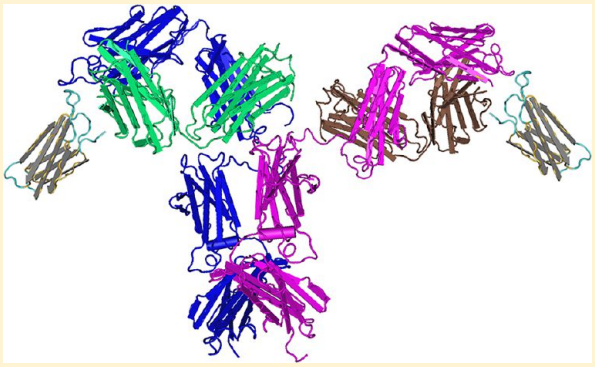
ABSTRACT: Mimetics of conformational protein epitopes have broad applications but have been difficult to identify using conventional peptide phage display. The 10th type III domain of human fibronectin (FNfn10) has two extended, randomizable surface-exposed loops and might be more amenable to the identification of such mimetics. We therefore selected a library of FNfn10 clones, randomized in both loops (15 residues in all), for binding to monoclonal antibodies (mAbs) that recognize the HIV-1 envelope glycoprotein. Anti-idiotypic monobodies (αIMs) mimicking both "linear" epitopes (2F5 and 4E10 mAbs) and conformational epitopes (b12 and VRC01 mAbs) were generated. αIMs selected against 2F5 and 4E10 frequently displayed sequence homology to the corresponding linear native epitopes. In the case of b12 and VRC01, we expected that the two constrained loop domains of FNfn10 would both contribute to complex conformational interactions with target antibodies. However, mutagenesis studies revealed differences from this simple model. An αIM selected against b12 was found to bind its cognate antibody via only a few residues within the BC loop of FNfn10, with minimal contribution from the FG loop. Unexpectedly, this was sufficient to generate a protein that engaged its cognate antibody in a manner very similar to that of HIV-1 Env, and with a strong K_D (43 nM). In contrast, an αIM selected against VRC01 engaged its cognate antibody in a manner that was dependent on both BC and FG loop sequences. Overall, these data suggest that the FNfn10 scaffold can be used to identify complex structures that mimic conformational protein epitopes.



Anti-idiotype antibodies provide powerful tools for studies of molecular mimicry and epitope topology,[1,2] as well as for biotechnology applications such as the specific detection and quantitation of therapeutic proteins in clinical settings.[3,4] The large physical size of antibody molecules, combined with constraints on their structure and diversity, has led to an interest in alternative approaches to developing anti-idiotypic reagents.

Conventional peptide phage display technology involving the display of short linear peptides on the surface of filamentous phage[5] has been successfully used to derive peptide mimics of linear virus-neutralizing antibody epitopes.[6,7] However, this approach has been less successful in developing mimics of nonlinear, conformational structures,[8] including mimics of the well-characterized HIV-1 broadly neutralizing antibody (bNAb), b12.[9,10] Constraining linear peptide sequences by flanking them with cysteine residues to generate a loop structure or embedding a loop structure within a stable scaffold has been used to improve the affinity of the peptides for their target antibody, presumably by reducing peptide flexibility.[11−13] This suggests that a protein scaffold with several randomizable loop domains might be proficient at making complex, conformationally dependent, interactions with target antibodies, and thereby facilitate the identification of conformational epitope mimetics.

To test this hypothesis, we performed a series of proof-of-concept experiments, using an alternative display scaffold based on the 10th fibronectin type III domain (FNfn10) of human fibronectin. FNfn10 is a small β-sandwich protein domain with a folding pattern similar to that of immunoglobulin[14,15] (Figure 1A); it has also been shown to exhibit a high level of physical stability and the ability to permit selection of ligand-binding proteins with nanomolar affinity.[15−17] The FNfn10 scaffold offers potential advantages over both the conventional, linear peptide display and antibody platforms. First, FNfn10 contains three adjacent structural loops (BC, DE, and FG) that can be diversified either alone or in combination, thereby creating the potential for the generation of both linear and discontinuous structures on the scaffold surface. Second, the

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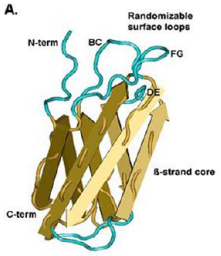
A.



B.

Library	Size	Potential Amino Acids Encoded
Lib NHK	~4 x 10^7	Tyr, Ser, Asp, Ala, Glu, Phe, Leu, Asn, Thr, Ile, Lys, Pro, His, Gln, Val, Met
Trimer	~1 x 10^9	All amino acids except Cys

Figure 1. FN structure and construction of randomized libraries. (A) Structural representation of the fibronectin scaffold. β-Strands A−G of the FNfn10 molecular scaffold are shown (yellow arrows), along with the three adjacent surface-exposed loops [BC, FG, and DE (turquoise)]. The structure was generated using CN3D, from the source data file MMDB IB 57520. (B) Randomized molecular libraries used in screening experiments.

suggested preference for monobodies to bind to residues found in protein−protein interacting surfaces coupled with their smaller size and more efficient expression would offer significant advantages relative to antibody-based reagents.[15,17]

In this work, we randomized the BC and FG loops of FNfn10 creating large peptide display libraries from which we selected anti-idiotypic monobodies (αIMs) capable of binding to a panel of well-characterized HIV-1 monoclonal antibodies (mAbs), including 4E10, 2F5, Z13e1, 447-52D, VRC-01, and b12. In this manner, we generated αIMs that mimicked both "linear" mAb epitopes (2F5, 4E10, Z13e1, and 447-52D) and conformational mAb epitopes (b12 and VRC-01). αIMs that mimicked the linear mAb epitopes frequently demonstrated sequence homology to the corresponding mAb epitopes within HIV-1 Env. In contrast, and as expected, αIMs that mimicked the conformational b12 and VRC-01 epitopes did not show sequence homology with HIV-1 Env. However, these αIMs specific for b12 were shown to engage the same contact residues that mediate binding of the b12 antibody to HIV-1 Env. This is in striking contrast to the previously described B2.1 peptide, a b12-binding peptide derived by conventional peptide display technology, which bound to the b12 antibody in a manner quite different from that employed by HIV-1 Env.[9,10,18] Additionally, αIMs that mimicked both discontinuous (b12) and linear (4E10 and 2F5) antibody epitopes were found to efficiently compete with native epitopes for binding to their cognate mAb.

Mutagenesis studies of two αIMs selected against conformation-dependent mAbs revealed distinct modes of interaction between αIMs and their cognate antibodies. In one case, binding was mediated by a small number of residues within the BC loop of FNfn10, with a minimal contribution from the FG loop. In the other case, binding of the αIM to its cognate antibody was dependent on both BC and FG loop sequences. Overall, these data suggest that the FNfn10 scaffold can be used to facilitate the discovery of complex structures that mimic conformational protein epitopes.

EXPERIMENTAL PROCEDURES

Generation of the Monobody Library. The FNfn10 scaffold was cloned into pAP-III6FL, a derivative of pAP-III6 containing a full-length gene III of M13, downstream of a FLAG epitope sequence. This plasmid was introduced into CJ236, an *Escherichia coli* strain that incorporates deoxyuracil rather than thymine, and single-stranded DNA (ssDNA) containing uracil was produced by infection with VCS M13 helper phage (Agilent); ssDNA from the phagemid particles was then purified using a QiaPrep Spin M13 kit (Qiagen). A derivative containing AseI restriction sites and in-frame stop codons (ATTAAT) within the BC and FG loops was generated by site-directed mutagenesis[19] and confirmed by sequence analysis. For NHK library construction, the template was annealed to mutagenic oligonucleotides targeting the BC (amino acids 23−29) and FG (amino acids 77−84) loops in large-scale reactions as described previously.[20] The library oligonucleotides (BC, 5′-CGTGATAC-GGTAATAACGMDNMDNMDNMDNMDNMDNMDNCCA-GCTGATCAGCAGGCT; FG, 5′-AATCGAGATTGGCTTGG-AMDNMDNMDNMDNMDNMDNMDNMDNAGTAACAG-CATATACAGTGATGGT) partially randomized seven positions in the BC loop and eight positions in the FG loop using NHK (N = A, C, G, or T; H = A, C, or T; K = G or T) codons. All amino acids except Arg, Trp, Cys, and Gly are encoded by using the NHK scheme. The template mix was electroporated into TG-1 cells (Agilent), and ~5 × 10^8 ampicillin resistant transformants were obtained. Colony polymerase chain reaction (PCR) followed by restriction digestion of the FNfn10 insert with AseI revealed that ~40% of the colonies obtained had incorporated both oligonucleotides, yielding a functional library size of ~2 × 10^8. The remaining unmutated or singly mutated clones will not display a monobody upon infection with helper phage and therefore are eliminated during the panning process.

The colonies were resuspended by scraping the plates with LB broth, and an aliquot containing >10 times the number of transformants in the library was subcultured and grown in LB medium at 37 °C for 2 h. Ten milliliters of this culture was infected with either VCS M13 helper phage (for monovalent display) for 2 h and diluted into 200 mL of LB containing ampicillin (100 μg/mL) and kanamycin (70 μg/mL). The culture was grown overnight at 30 °C, and phage from the supernatant was harvested by precipitation with polyethylene glycol, resuspended in 50 mM Tris-HCl (pH 7.5) and 150 mM NaCl (TBS) containing 0.5% casein and 15% glycerol, and frozen in aliquots at −80 °C.

Libraries generated using triphosphoramidites (Glen Research, 19 codon mix, no cysteine) were prepared using a derivative of pAP-III6 containing the Fnfn10 scaffold with the *phoA* promoter and FNfn10-truncated gene III fusion protein region inverted to generate ssDNA complementary to the sense strand trimer-containing oligonucleotides [Tri BC, 5′-CTGA-TCAGCTGG(Tri)$_7$CGTTATTACCGT; Tri-FG, 5′-TACGC-TGTTACT(Tri)$_8$TCCAAGCCAATC, where Tri indicates the 19-codon mix]. The trimer library oligonucleotides were obtained from the W. M. Keck Oligonucleotide Synthesis Facility at Yale University (New Haven, CT). Large-scale mutagenesis and library generation were as described above. A total of ~1 × 10^9 doubly mutated clones were obtained from five large-scale electroporations.

Library Selection on mAbs. Target mAbs (b12, 447-52D, Z13e1, 4E10, 2F5, and VRC-01) were obtained from the AIDS Reagent Repository;[21−36] Rtx (Rituxan; specific for the B cell surface protein, CD20[37]), 1F1 (specific for Dengue virus; kindly provided by J. J. Schlesinger, University of Rochester, Rochester, NY[38]), and F10 (specific for influenza virus hemagglutinin[39]) were used as irrelevant controls. Note that the final F10 antibody used for our FN selections corresponded to a pairing of the heavy chain from the F10 antibody[39] with an arbitrary λ chain and is therefore designated F10λ.

Antibodies were immobilized in microtiter dish wells at 50 μg/mL in TBS overnight, blocked with TBS containing 0.5% casein. Aliquots of the libraries diluted 1:1 with TBS and casein were added to two wells (50 μL/well), and the plate was

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shaken for 2 h at room temperature. Phage were removed, and the wells were washed with TBS and 0.5% Tween 20 seven times over 20 min followed by one wash with water. Bound phage were eluted with 0.1 M glycine hydrochloride (pH 2) and 0.1% bovine serum albumin for 15 min. The eluate was removed, neutralized with Tris base, transduced into midlog TG-1 cells, and plated on LB plates containing ampicillin. The next day, colonies were scraped from plates in 5 mL of LB and subcultured into fresh medium for the production of the next round of phage. Aliquots (1 mL) of the midlog culture were infected with VCS M13 helper and grown as described above. Second and third rounds of phage were prepared from 30 mL cultures as described above, and 50 mL aliquots were applied to single wells coated with the target mAb. Individual clones after the second or third round of enrichment were tested by phage ELISA to confirm binding to the target mAb and lack of reactivity to an irrelevant myeloma IgG1 protein. The FNfn10 inserts of positive clones were amplified via PCR using flanking vector primers and sequenced.

Production and Purification of αIMs. Selected FNfn10 variants were subcloned into a pET22 vector (Novagen) by introducing a methionine codon in front of the FLAG epitope as part of an NdeI restriction enzyme site. The carboxy terminus of FNfn10 was modified to add a *birA*[13] site, and the modified FNfn10 was cloned between the NdeI and XhoI sites of the vector, adding a His6 purification tag immediately following the *birA* site. Cultures were grown in LB to midexponential phase, and protein expression was induced for 2 h by addition of 1 mM IPTG and 50 μM biotin. Cells were harvested and lysed with Bugbuster reagent (Novagen) and purified by nickel affinity chromatography using Ni^{2+} magnetic beads and a Thermo Kingfisher instrument. Proteins were eluted from the beads with PBS containing 250 mM imidazole and stored at 4 °C. The purity of the proteins was determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and Simply Blue staining (Invitrogen). For some experiments, the protein was dialyzed into PBS before use. We verified the functionality of the biotinylated αIMs by comparing the titer of the proteins after capture on streptavidin-coated wells using both anti-Flag (Sigma) and the cognate antibody to the αIM.

Generation of b12 mAb Mutants. The b12 mAb and alanine substitution mutants were generated by cloning synthetic fragments encoding the variable regions of b12 (GeneArt) into vectors for mammalian cell expression described previously.[40] The wild-type variable heavy chain (VH) and Y53A and Y98A mutants were directly cloned into the heavy chain expression vector from the synthetic genes. The W100A mutant was generated from a single-chain antibody fragment variable (scFv) construct by site-directed mutagenesis as described above, and the mutant VH region was amplified via PCR and cloned into the mammalian expression vector. Each VH variant along with wild-type b12 VH was cotransfected with the b12 variable light chain (VL) plasmid into HEK293 cells using FuGene (Roche), and the supernatant was harvested 3 and 6 days post-transfection. Antibodies were purified from the culture supernatant using protein G magnetic beads (Thermo) and processed on a Thermo Kingfisher instrument. Antibodies were eluted from the beads in 0.1 M glycine hydrochloride (pH 3), neutralized with Tris base, and dialyzed into PBS.

The production of the b12 Fab was accomplished by modifying the heavy chain expression vector by deleting the hinge, CH2, and CH3 domains and replacing them with a hexahistidine tag. Five days post-transfection, the cell culture supernatants were collected and centrifuged at 3500*g* to remove cell debris. Fab protein was purified by metal affinity chromatography using Ni-NTA resin (Qiagen). Columns were washed with increasing concentrations of imidazole (20 and 40 mM) followed by elution in the presence of 250 mM imidazole. Fractions containing purified protein were pooled and dialyzed against PBS.

Alanine-Scanning Mutagenesis. A representative b12 mAb-binding αIM, b12 3-5 αIM, was chosen for further characterization. Single-alanine mutations at all positions of the b12 3-5 αIM BC and FG loops (except for position 26, which is Ala in wild-type b12 3-5 αIM) were generated using site-directed mutagenesis of the b12 3-5 αIM sequence as described above. Individual biotinylated variant proteins were prepared from pET clones as described above and immobilized on streptavidin-coated wells using a saturating amount of bio-αIM. A nonsaturating dilution of the b12 mAb was prepared and added to all wells in an ELISA. After incubation with the anti-human IgG−HRP conjugate, bound antibody was detected after incubation with TMB substrate. In addition to alanine substitution mutants, two hybrid FNfn10 proteins were also evaluated. Each retained the b12 3-5 αIM BC loop sequence, but we exchanged the FG loop for those found in the Rtx and 1F1 αIMs. These hybrid clones were generated by swapping the C-terminal half of the genes using the unique EcoRI restriction site present between the BC and FG loops of the scaffold. For confirmation of the specificity of the b12 mAb against our b12 αIM, an ELISA (described below) was performed after wells had been passively coated with b12 3-5 αIM, 4E10 αIM, 1F1 αIM, and RTX αIM. b12 mAb was added at a concentration of 150 μg/mL followed by goat anti-human IgG conjugated to HRP at a 1:1000 dilution. After the sample had been washed, HRP substrate (TMB) was added, color was allowed to develop, and the plate was photographed. The reaction was stopped by the addition of 50 μL of 1 M phosphoric acid, and the optical density was read at 420 nm.

Generation of VRC-01 and 447-52D Mutants. Loop swap hybrid αIMs of VRC-01 3-2, VRC-01 3-11, VRC-01 3-13, 447-52D J2, and 447-52D J3 were generated as described above. Hybrids retained the original wild-type BC or FG loop, while the reciprocal loop was swapped for an all-serine substitute. The BC loop hybrids, FG loop hybrids, and parental αIMs were analyzed by a phage ELISA (described below).

The VRC-01 3-2 αIM was mutated by a variant of combinatorial alanine-scanning mutagenesis[41] whereby all non-serine residues within the BC and FG loops were restricted to either the wild-type amino acid or serine, with the exception of positions encoding Met, His, or Arg where it was necessary to have four amino acids represented. The oligonucleotides used to generate the serine scan library were 5′-CTGCTGATCAGCTGGTYGCGMRTKCGYC-TAKSKCTCGTTATTACCGTATC (BC) and 5′-GTATACGC-TGTTACTYCGTYTTMTAGTTYTYCTMRTAKSTCCAAGC-CAATCTCG (FG), where K = G or T, M = A or C, R = A or G, Y = C or T, and S = C or G. The annealed and extended template mixture was electroporated into TG1, and ~1×10^8 transformants were obtained. After infection with helper phage, the displayed phage library was panned against anti-FLAG or VRC-01 mAb.

ELISA Analysis. *Phage ELISA.* Phage were prepared by PEG precipitation from 1.2 mL cultures using VCS M13 helper and resuspended in 0.3 mL in TBS and 0.5% casein, and 50 μL was added to the wells. After incubation for 1 h at room temperature, the wells were washed 10 times and a 1:2000 dilution of the anti-M13−HRP conjugate was added for a 1 h

incubation. After the sample had been washed, HRP substrate (TMB) was added, color was allowed to develop, and the plate was photographed. The reactions were stopped by addition of 50 μL of 1 M phosphoric acid, and the optical density was read at 420 nm.

Antibody ELISA. Purified αIMs were diluted in TBS and casein to a final concentration of ~1 μg/mL and captured on streptavidin-coated wells. After the samples had been washed with TBS, serial half-log dilutions of selected mAbs were added to each well and incubated for 1 h at room temperature. Addition of the secondary goat anti-human IgG–HRP antibody (KPL, Gaithersburg, MD) was followed by HRP substrate (TMB) as described above.

b12 Competition ELISA. Purified, biotinylated b12 3-5 αIM was bound to streptavidin-coated wells and washed with PBS and 0.05% Tween (PBS-T). Serial 2-fold dilutions of oligomeric YU2gp140[42] or the YU2gp140 D368R mutant, from 0.4 to 25 μg/mL, were preincubated with a fixed amount of IgG1 b12 mAb (1 μg/mL) at 37 °C for 30 min, before being added to b12 3-5 αIM-coated wells for 2 h at room temperature. After washes with PBS-T, a secondary anti-human IgG–HRP antibody was added at a 1:5000 dilution for 1 h at room temperature. Following five washes, the ELISAs were developed with 100 μL of TMB substrate. The reactions were stopped by adding 100 μL of 1 M sulfuric acid to each well, and the optical density at 450 nm was read. Each condition was repeated in duplicate.

2F5 and 4E10 Competition ELISA. Peptides corresponding to the 2F5 and 4E10 epitopes from HIV-1 gp41 were synthesized and conjugated to BSA (2F5, CNEQELLELDKWASLWSGGRGGL; 4E10, CSLWNWFDITNWLWRRK).[25−28] The BSA-conjugated peptides were then coated at a concentration 5 μg/mL overnight onto polystyrene wells. A serial half-log titration of the respective mAb was added sequentially to each well and developed as described in Antibody ELISA. A titration curve was created to determine the optimal concentration of mAb for the competition ELISA. The 2F5/4E10 peptide conjugated to BSA was coated overnight at a concentration of 5 μg/mL in polystyrene wells. After being washed five times with TBS, the plates were blocked with Blocker TM for 2 h at room temperature. 2F5 mAb (50 ng/mL) was preincubated with half-log dilutions of 2F5 αIM, MPER peptide, or F10λ αIM protein. 4E10 mAb (15.8 ng/mL) was preincubated with half-log dilutions of 4E10 5-2 αIM, 4E10 5-5 αIM, MPER peptide, or F10λ αIM protein. Alternatively, a 1:400 dilution of sera from either an HIV-1-positive person (subject 026) or an HIV-1-negative person (subject 1609) was preincubated with half-log dilutions of 4E10 5-2 αIM, 4E10 5-5 αIM, MPER peptide, or F10λ αIM before addition to the coated plates. After the samples had been extensively washed with TBS, mAb bound to the αIMs or peptide was detected with the goat anti-human IgG–HRP conjugate at a 1:2000 dilution. HRP substrate (TMB) was used for the development of the assay, and the reactions were stopped by addition of 50 μL of 1 M phosphoric acid to each well after an image had been acquired. At this point, the optical density was read at 420 nm.

Detection of Rituximab in Pooled Normal Sera. Biotinylated Rtx αIM or b12 3-5 αIM was coupled to neutravidin microtiter wells at a concentration of 1 μg/mL in triplicate. Rituximab was serially diluted in half-log increments in blocking solution containing 1% pooled normal sera, starting at a concentration of 10 μg/mL. After the samples had been extensively washed, goat anti-human IgG conjugated to HRP was added at a 1:1000 dilution. HRP substrate (TMB) was added, and color was allowed to develop. The wells were photographed, and the reaction was stopped by the addition of 50 μL of 1 M phosphoric acid to each well. The optical density was recorded at 420 nm.

Collection of Human Serum Samples. Peripheral blood samples were obtained from HIV-1-positive persons at the University of Rochester Medical Center and the University of Washington (Seattle, WA) HIV clinics between 2004 and 2010. Samples from healthy control subjects were obtained at the University of Rochester. All subjects provided signed written informed consent. All procedures and methods were approved by the University of Rochester Research Subjects Review Board and the University of Washington Institutional Review Board.

SPR Kinetic Binding Analysis. The binding affinity of b12 3-5 αIM for the b12 Fab was determined by surface plasmon resonance using a Reichert SR7500DC SPR instrument.

Table 1. Summary of Anti-Idiotypic Monobody Selections on Monoclonal Antibodies[a]

target mAb	library[b]	no. of rounds	ELISA positive	no. of unique sequences
b12	NHK, Tri	3	20/23	11
4E10	Tri	3	33/34	9
1F1	Comb NHK and Tri	3	6/6	3
2F5	Comb NHK and Tri	4	8/24	2
Z13e1	Tri	3	12/15	9
447-52D	NHK-HP and Tri	2	16/16	16
VRC-01	Comb NHK	3	8/16	7
Rituxan	Tri	3	18/20	7

[a]All enrichments were performed for three rounds on the indicated target mAb (coated at a concentration of 50 μg/mL in microtiter plate wells) except for the 447-52D enrichment, which was stopped after two rounds when high-level enrichment was already evident. [b]The following libraries were used: NHK, produced by infection with VCS helper; Tri, trimer library produced by infection with VCS helper; Comb NHK and Tri, an equal volume mixture of the two VCS-produced libraries; NHK-HP and Tri, NHK library infected with helper phage and combined with an equal volume of the trimer library. Individual binding clones after two or three rounds of enrichment were identified by phage ELISA, and the αIM inserts were amplified by colony PCR and sequenced.

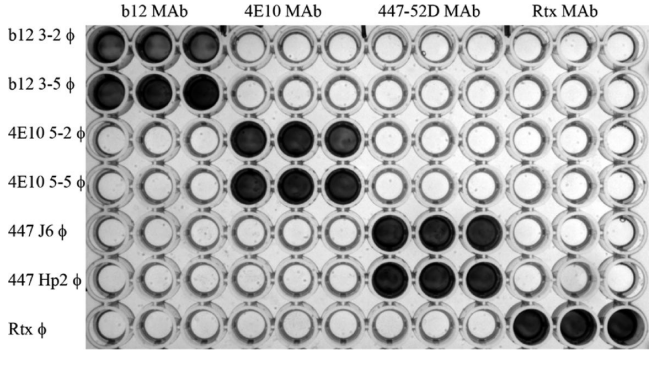

Figure 2. ELISA analysis of the binding specificity of epitope mimics displayed on phage. The indicated antibodies (top axis) were immobilized in triplicate in plate wells, and phage clones displaying the indicated αIMs (left axis) were then added to the wells. After the samples had been washed, bound phage was detected using the anti-M13–HRP conjugate. It can be readily appreciated that each of the phage clones reacted specifically with its cognate antibody (top axis), but not with irrelevant or unrelated antibodies.

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Table 2. Sequence Analysis of Representative Anti-Idiotypic Monobodies[a]

clone	BC loop	FG loop	clone	BC loop	FG loop
b12			Z13		
FN3-2	VHFALPV	TNHYMV	Tri-Z13-3-1	QPTFMPE	MIP**W**MVPG
FN3-5	VHFALPV	HISHQHIL	Tri-Z13-3-2	EWQVDAE	D**W**W**W**DTIE
Tri-3-1	VHFAWTV	NWGDTHQH	Tri-Z13-3-3	MEYSWQY	P**WNW**V**D**L**T**
Tri-3-6	VHWALPV	WWSGQWMP	Tri-Z13-3-4	ERWAMWY	HPWIKWYW
Tri-3-8	VHFAYPA	DITMGYMY	Tri-Z13-3-5	AHAWLPE	WHP**W**FQYE
Tri-3-9	MDHTWLP	QWDQNVWP	Tri-Z13-3-7	QEASWLS	WVPEQFDQ
2F5			Tri-Z13-3-9	YDEWFWT	DPWLMPPA
Tri-2F5-15	MW**D**K**W**SY	WWIGEFPV	Tri-Z13-3-11	RRFWPPF	TATKSFWW
Tri-2F5-17	PY**D**K**W**A**Y**	RWYWVPHY	Tri-Z13-3-14	SSTEWFV	DIPKQWGK
			447-52D		
4E10			Hp1	TVHAVPT	LYPLDQSS
Tri-5-2	TEWPEQY	RHLFEYAE	Hp2	SANFPSV	DLHFYVVS
Tri-5-5	RWDPFTH	Q**NW**F**N**Q**T**S	Hp3	ANAPVAT	LYPMQASL
Tri-2-6	DAHSFTA	W**NW**F**E**Q**T**N	Hp4	SKSTFHV	TSYYKKFP
4E10-3-3	DAPAVTV	H**N**F**F**AQ**S**A	Hp5	LAPLSVV	LYPYPVAS
4E10-3-5	DAPAVTV	VLTHNFNN	Hp6	QFQYPVV	STYPLLIF
4E10-3-6	SPLTFPA	F**N**F**F**LQ**T**A	Hp7	APPLNVV	LYPKPEPH
			Hp8	APSLPVV	LYPFPTQD
VRC01			J1	FPSSLPV	LYNPQYMY
3-1	SPPTFPM	SLLDFPIV	J2	STSPLPT	LYPSHLSS
3-2	LPHAPMA	PFYSFPHM	J3	PPVMSPV	LYPPAKVH
3-5	MPFVFTP	SYVPFAD	J4	LPPPHPL	LYPYPPIS
3-10	LHHSPSA	LTLLPNYV	J5	YVTLHPV	LYPYPTHL
3-11	HISFPYT	SLFYPVPS	J6	TLPMLPT	LYPYPYEY
3-13	ATSLSTL	FPNYSYPL	Tri-J7	NWRWVMED	**SIHMGPRG**
3-16	ASSLPSL	FAPQLHTL	J8	PATQHPV	LYPLPPLL
Rituxan	PKLGVNK	V**WEW**DQPQ	1F1	MHQMPWV	YWRTTPFM

[a]Clones designated Tri-*X*-*Y* correspond to clones derived from the trimer library. All other clones were derived from the NHK library. All clones were isolated and sequenced after three iterative cycles of panning against the target antibody except for the 447-52D αIMs, which were characterized after two rounds. Bold residues indicate partial homology to the reported linear epitopes for 4E10 (NWFNIT), Z13e1 (WNWFDITN), 447-52D (SIHXGPXX), and 2F5 (ELDKWAS). For Rituximab binders, bold residues indicate partial homology with a mimotope sequence previously isolated from a cyclic peptide display library (WWEWS/T).[2]

Streptavidin was immobilized via standard amine coupling onto a planar mixed self-assembled monolayer consisting of 10% carboxyl groups in a polyethylene glycol background. The surface was first activated with a mix of 0.04 mg/mL 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) (Pierce) and 0.01 mg/mL *N*-hydroxysuccinimide (NHS) (Sigma) to form NHS esters on the surface. Streptavidin (200 μg/mL) in 10 mM sodium acetate was injected over both the sample and reference channels. Two thousand micro refractive index units of streptavidin was immobilized. The surface was then blocked by injecting 1 M ethanolamine hydrochloride (pH 8.5) for 10 min; 470 micro refractive index units of biotinylated αIM was then captured on the sample channel only. Association and dissociation data were collected by injecting dilutions of the Fab (in PBS and 0.05% Tween 20) over the sample and reference channels at 50 μL/min in duplicate. Data overlays were performed using Scrubber 2.0a (Biologic Software Pty., Campbell, Australia). The data were double referenced using the reference channel along with buffer blank injections. Overlays were exported to an SPR data analysis program[43] that used Bayesian analysis for determination of binding constants. The association phase of each sample injection was fit to a single-exponential growth equation using GraphPad Prism (GraphPad Software Inc.) to determine the equilibrium binding level. The calculated equilibrium for each injection was plotted versus concentration and fit to a Langmuir binding isotherm {response = equilibrium response × $[(K_A[\text{b12 Fab}])/(K_A[\text{b12 Fab}] + 1)]$} to determine the equilibrium binding constants.

RESULTS

Generation of Anti-Idiotypic Monobodies. We generated two distinct libraries of randomized BC and FG loops displayed on the FNfn10 scaffold (Figure 1). These libraries were then selected against a variety of target monoclonal antibodies (mAbs), including several with broadly neutralizing activity against HIV-1, one murine mAb, and a murine-derived therapeutic mAb. Selections against the mAbs were generally completed after three iterative cycles of panning, and the majority of the selected population consisted of clones that were positive for mAb binding as determined by phage ELISA (Table 1). We found no αIMs that cross-reacted with other mAbs, as might have been expected because all of the human mAb targets were of the IgG1 isotype. However, this result is consistent with the observations of Koide and co-workers,[44] who have suggested that the FNfn10 monobodies have a predilection for binding to surfaces involved in protein−protein interactions (such as antibody combining sites).

Individual clones were identified as binders by phage ELISA, and the sequences of their BC and FG loops were then determined. In nearly all enrichments, we did not screen exhaustively, so the number of clones reported in Table 1

represents a minimal number, particularly for those enrichments that yielded a large number of binding clones. We also evaluated a subset of the selected phage for their reactivity with nonselecting mAbs. As shown in Figure 2, individual clones reacted only with their cognate mAb and had no reactivity with any nonselecting antibody. We determined the sequence of all positive clones, and examples of the results are listed in Table 2. As might be anticipated, some of the αIMs selected against target antibodies that recognize mainly linear epitopes (2F5, 4E10, 447-52D, and Z13e1) were partially homologous in their amino acids to the reported epitopes recognized by these mAbs.

Characterization of Anti-Idiotypic Monobodies for Critical Binding Residues. The b12 mAb is a well-characterized broadly neutralizing antibody that recognizes a discontinuous epitope within the CD4 binding site of HIV-1 Env.[21−24] Previous studies have shown that b12 mAb residues Y53, Y98, and W100 are essential for binding to HIV-1 gp140.[10] We therefore examined the reactivity of our b12-binding phage clones with wild-type b12 mAb and with Y53G, Y98A, and W100A mutants of b12. As a positive control for these experiments, we used the B2.1 peptide phage clone described by Scott and co-workers (kind gift of J. K. Scott, Simon Fraser University, Burnaby, BC). This phage clone was selected from a conventional peptide display library[36] and does not bind to the b12 mAb in the site where gp120 binds.[9,45] As a consequence, some mutations in the b12 mAb can differentiate this peptide from gp120, including mutations of Y53, Y98, and W100.[9,18] We therefore used these mutations to assess whether our b12-reactive monobodies bind in the gp120 binding site, as opposed to the B2.1 peptide binding site (which is on the other side of CDR-H3 from the gp120 binding site). As shown in Figure 3, all of the b12-specific αIM clones tested bound to the

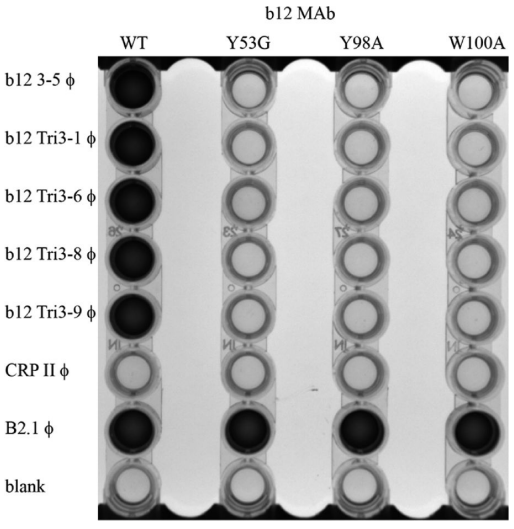

Figure 3. ELISA analysis of the specificity of selected b12 reactive phage clones. The indicated b12 antibodies [wild-type or mutant (top axis)] were immobilized in triplicate in plate wells, and phage clones displaying the indicated αIMs (left axis) were then added to the wells. After the samples had been washed, bound phage was detected using the anti-M13−HRP conjugate. B2.1 is a positive control phage encoding a peptide mimotope that binds wild-type b12 mAb as well as the three mutant forms of the b12 mAb.[9,18] CRPII is an irrelevant phage clone, not selected against the b12 mAb.

wild-type b12 mAb but failed to bind to the Y53G, Y98A, and W100A b12 mutants. Thus, the b12 αIMs bind in a distinctly different mode to the mAb than the B2.1 peptide and require the same residues of the b12 paratope that are necessary for binding to gp120.

We next produced soluble recombinant protein corresponding to a subset of the phage clones. αIM-encoding sequences from phage clones of interest were subcloned into a prokaryotic expression vector and expressed in *E. coli* BL21 host cells with terminal His, biotin, and FLAG tags. Proteins were then purified using metal affinity chromatography to bind the protein His tag. Samples of our purified b12 3-5 αIMs were then analyzed by SDS−PAGE and visualized by staining with Coomassie Blue (Simply Blue) dye (Figure 4A). A single

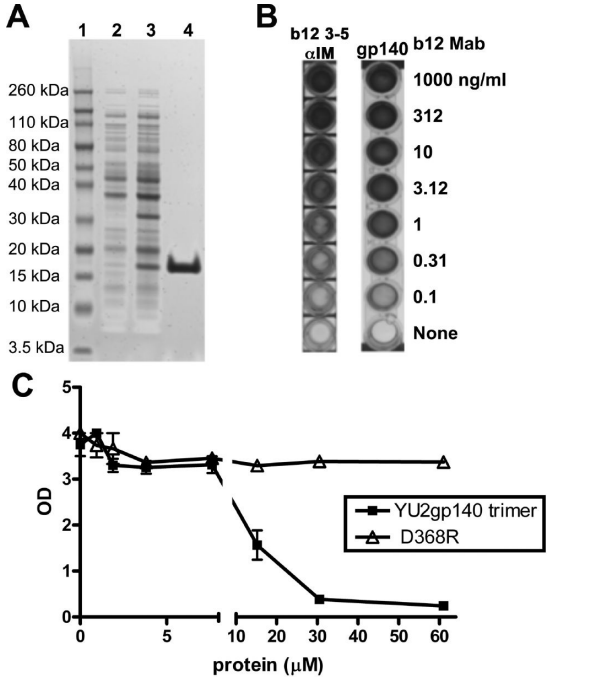

Figure 4. Binding of purified b12 αIMs to the b12 mAb is competitively inhibited by HIV-1 Env. (A) The b12 3-5 αIM was expressed in *E. coli* BL21(DE3) cells. A 4 to 12% Bis-Tris gel was loaded with samples of the uninduced culture (lane 2), induced culture (lane 3), and the purified eluate collected after column purification of the b12 αIM (lane 4); lane 1 contained molecular mass markers. (B) ELISA analysis of the binding of serially diluted b12 mAb to biotinylated b12 3-5 αIM (5 μg/mL) and HIV-1 Env gp140 oligomers.[42] (C) b12 mAb (1 μg/mL) was preincubated with varying amounts of wild-type HIV-1 Env gp140 oligomers or mutant gp140 oligomers (D368R; this lacks the ability to bind to b12) and then added to the wells coated with b12 3-5 αIM. The b12 3-5 αIM competitively inhibits binding of the b12 mAb to HIV-1 Env gp140 oligomers.

protein species with the expected molecular mass (15−17 kDa) was detected. Subsequently, we tested the ability of this protein to bind the b12 mAb. As shown in Figure 4B, the b12 3-5 αIM bound efficiently to immobilized b12 mAb, as did HIV-1 gp140,[42] the positive control for the assay. We further examined the ability of HIV-1 gp140 to competitively inhibit binding of the b12 mAb to immobilized b12 3-5 αIM. An HIV-1 gp140 mutant that is unable to bind to the b12 mAb was used as a control (D368R).[46] The results (Figure 4C) show that wild-type gp140, but not the D368R mutant, competitively inhibited binding of the b12 mAb to our b12 3-5 αIM protein.

To determine which residues of the αIM were important for antibody binding, we performed a systematic alanine

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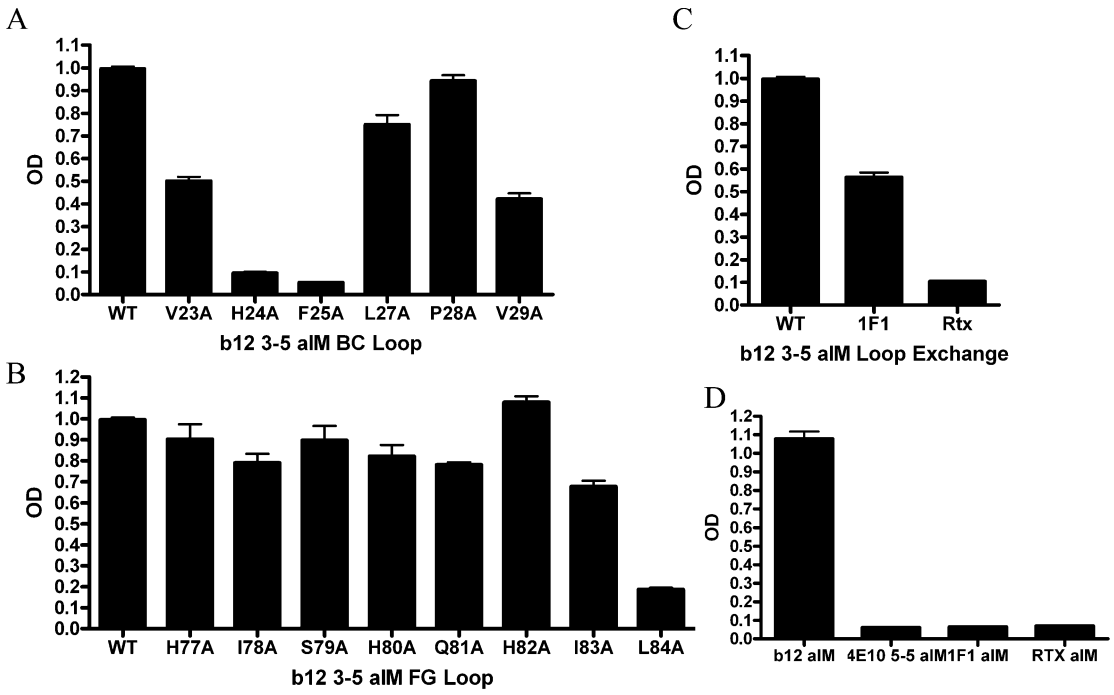

Figure 5. Contribution of BC and FG loop residues to b12 mAb binding by the b12 3-5 αIM. αIMs, including wild-type b12 3-5 αIM and mutated derivatives thereof (as indicated), were captured on ELISA plates. b12 mAb was then added, and antibody binding was detected with the anti-human IgG–HRP conjugate. (A) For the wild-type b12 3-5 αIM sequence, the BC loop sequence is VHFALPV and (B) the FG loop sequence is HISHQHIL. A library of single-alanine mutants for each of the amino acids in both the BC and FG loops was created and analyzed for b12 binding. (C) The FG loop of the wild-type b12 3-5 αIM was replaced with FG loops from irrelevant αIMs: 1F1 (FG loop, YWRTTPFM) or Rtx (FG loop, VWEWDQPQ). (D) Wells were coated with αIM as indicated, and an ELISA was performed with b12 mAb; the data indicate that mAb binding was specific to the b12 3-5 αIM and did not occur with irrelevant αIMs, selected against different antibody targets (1F1, Rtx, and 4E10).

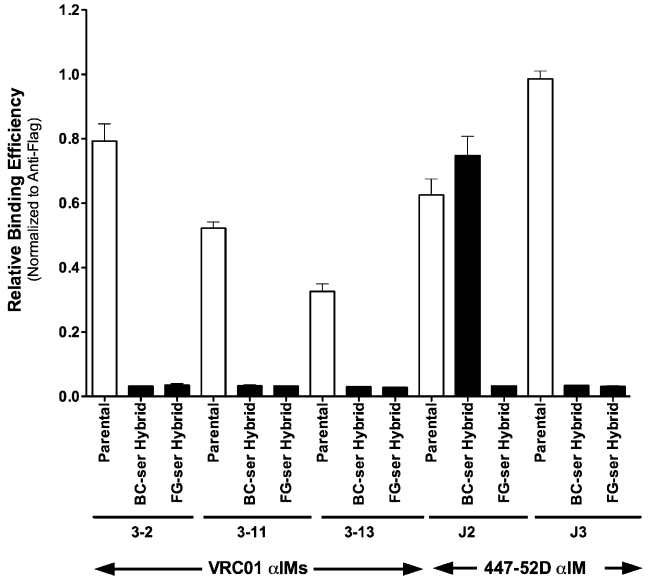

Figure 6. Contribution of BC and FG loops to the binding of VRC01 and 447-52D IMs to their cognate antibodies. VRC-01 3-2, 3-11, 3-13, and 447-52D J2 and J3 αIMs were mutated, replacing either the full BC or FG loop with serine residues. Binding of the resulting hybrids to their cognate mAb was then assessed, and results were compared to binding to the corresponding parental αIMs. In all cases, except 447-52D J2, substitution of either the BC or FG loop with serine residues resulted in abrogation of binding of the αIMs to their cognate antibodies.

substitution mutagenesis of the BC and FG loop sequences and then tested each variant for its ability to bind the b12 mAb by an ELISA. As shown in Figure 5A, BC loop residues H24 and F25 appear to play a critical role in the recognition of the b12 mAb as they weakened binding by at least 10-fold. In contrast, Figure 5B shows only FG loop residue L84 contributed significantly to b12 mAb recognition, as reflected by the approximately 5-fold reduced level of binding by the L84A mutant. We conclude that the primary interaction between the b12 3-5 αIM and its cognate antibody is mediated through the engineered BC loop domain (notably, residues H24 and F25) and that the FG loop contributes more modestly to mAb binding (notably, residue L84).

We also conducted an additional experiment to examine the role of the FG loop in b12 mAb binding by the b12 3-5 αIM. To do this, the FG loop of the b12 3-5 αIM was exchanged for FG loop sequences from two irrelevant control αIMs (1F1 and Rtx). When the FG loop of the b12 3-5 αIM was exchanged for the corresponding loop from the Rtx αIM, binding to the b12 mAb was almost completely abrogated. However, binding was only modestly affected when the FG loop was exchanged for the corresponding domain from the 1F1 αIM (Figure 5C). The profound inhibitory effect of the Rtx FG loop domain is consistent with a role for the FG loop in binding to the b12 mAb, but the modest effect of the 1F1 FG loop sequence raises the possibility of an alternative interpretation, that the Rtx FG loop may have introduced a new, disruptive, or sterically hindering group. Further studies will be necessary to fully resolve this issue.

As an additional control, we examined the ability of αIMs selected against other mAbs to bind to the b12 mAb. As expected, only the b12 3-5 αIM (and not other recombinant αIMs, selected on different antibody targets) was reactive with b12 (Figure 5D).

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Table 3. Mapping of VRC-01 3-2 αIM Residues Required for Antibody Binding by Site-Directed Mutagenesis[a]

	BC LOOP							FG LOOP									BC LOOP							FG LOOP							
WT	**L**	**P**	**H**	**A**	**P**	**M**	**A**	**P**	**F**	**Y**	**S**	**F**	**P**	**H**	**M**	**WT**	**L**	**P**	**H**	**A**	**P**	**M**	**A**	**P**	**F**	**Y**	**S**	**F**	**P**	**H**	**M**
VRC01 SLXN:																**M2 SLXN:**															
V1	S	P	H	A	P	R	A	P	F	Y	S	F	P	H	M	*M2-2*	S	P	R	A	P	R	A	P	S	S	S	F	P	S	R
V7	L	P	H	S	P	S	A	P	F	Y	S	F	P	H	M	*M2-3*	S	S	H	S	P	M	S	P	F	Y	S	S	P	N	R
V2.3	S	P	H	A	P	R	A	S	F	Y	S	F	P	H	M	*M2-4*	L	S	H	S	P	M	S	P	S	Y	S	S	S	S	S
V1-11	S	P	H	A	P	R	A	P	F	Y	S	F	P	H	M	*M2-7*	S	S	H	S	P	R	S	S	S	S	S	S	P	S	S
V1-13	S	P	H	A	P	S	A	P	F	Y	S	F	P	H	M	*M2-8*	S	P	N	A	P	M	S	P	S	S	S	S	S	H	R
V1-14	S	P	H	A	P	S	A	P	F	Y	S	F	P	H	M	*M2-9*	L	P	R	S	P	R	A	P	S	Y	S	S	S	S	S
V1-21	S	P	H	S	P	M	A	P	F	Y	S	F	P	H	M	*M2-10*	S	P	N	A	P	S	S	P	S	Y	S	S	P	S	R
V1-22	S	P	H	S	P	S	A	P	F	Y	S	F	P	H	M	*M2-11*	L	P	N	S	S	S	A	S	F	S	S	S	S	S	R
V1-25	S	P	H	S	P	R	A	P	F	Y	S	F	P	H	M	*M2-12*	S	S	R	A	S	M	A	P	S	Y	S	S	S	H	R
V1-27	L	P	H	S	P	R	A	P	F	Y	S	F	P	H	M	*M-1*	S	S	N	A	S	R	A	S	F	Y	S	S	P	R	R
V1-33	L	P	H	A	P	M	A	P	F	Y	S	F	P	H	M	*M-2*	S	S	S	S	P	M	A	S	S	Y	S	Y	P	N	S
V-1	S	P	H	A	P	S	A	P	F	Y	S	F	P	H	M	*M-3*	L	P	S	S	P	S	A	S	S	S	S	F	S	N	R
V-2	S	P	H	A	P	M	A	P	F	Y	S	F	P	H	M	*M-4*	S	P	N	A	S	S	A	P	S	Y	S	S	S	S	R
V-3	S	P	H	A	P	R	A	P	F	Y	S	F	P	H	M	*M-5*	L	P	N	A	S	M	A	P	F	S	S	S	S	N	R
V-4	S	P	H	A	P	I	A	P	F	Y	S	F	P	H	M	*M-6*	S	P	R	A	S	M	A	P	F	Y	S	S	P	R	S
V-5	S	P	H	A	P	M	A	P	F	Y	S	F	P	H	M	*M-7*	L	P	H	A	S	I	A	S	S	S	S	S	S	H	R
V-6	L	P	H	S	P	R	A	P	F	Y	S	F	P	H	M	*M-8*	S	S	S	S	S	M	A	S	F	S	S	F	P	H	R
V-7	S	P	H	A	P	M	A	P	F	Y	S	F	P	H	M	*M-9*	S	S	H	A	S	I	A	P	S	S	S	F	P	R	R
V-8	S	P	H	A	P	R	A	P	F	Y	S	F	P	H	M	*M-10*	L	S	H	A	P	M	S	P	S	Y	S	S	P	H	R
V-9	S	P	H	S	P	R	A	P	F	Y	S	F	P	H	M	*M-11*	L	S	S	S	P	I	S	P	S	Y	S	S	P	R	R
V-10	S	P	H	A	P	S	A	P	F	Y	S	F	P	H	M	*M-12*	S	P	H	A	S	S	A	P	S	S	S	F	P	N	R
V-11	S	P	H	S	P	R	A	P	F	Y	S	F	P	H	M	*M-13*	S	P	N	S	P	M	A	S	F	Y	S	S	P	S	M
V-12	S	P	H	A	P	R	A	P	F	Y	S	F	P	H	M	*M-14*	S	P	N	A	P	M	S	S	F	Y	S	S	P	S	M
																M-15	S	S	N	A	S	M	A	P	S	S	S	F	P	H	S
VRCO1 CONS.		**P**	**H**		**P**		**A**	**P**	**F**	**Y**	**S**	**F**	**P**	**H**	**M**	**M2 CONS.**											**S**				

[a]Site-directed mutagenesis was used to create a minilibrary in which each position in the BC and FG loops of the VRC01 3-2 αIM was permitted to be either the wild-type sequence or a serine residue (except in the case of methionine and histidine, which were replaced with either serine, asparagine, arginine, or isoleucine). The resulting library was then selected for binding to either the VRC-01 mAb (left) or the irrelevant M2 anti-FLAG antibody (right). A selection of clones, 23 panned on VRC-01 and 24 panned on anti-FLAG, were sequenced; results are presented. Wild-type VRC-01 sequences are unshaded, while substitutions are color-coded by amino acid (e.g., red for serine). Sequences of the clones selected against the VRC-01 3-2 αIM demonstrate the critically important role of the complete FG loop and four of seven residues within the BC loop in contributing to the binding of the VRC-01 3-2 αIM to its cognate antibody. In contrast, sequences of the clones selected against the irrelevant M2 anti-FLAG antibody reveal the diversity of the parental minilibrary.

The analysis of the b12 3-5 αIM mutant revealed that, unexpectedly, most of the key residues were in the BC loop. To determine whether this was a generalizable finding, we conducted additional "loop exchange" experiments using αIMs that were reactive with two other mAbs: (i) the VRC-01 mAb (which recognizes a conformational epitope in the CD4-binding site of HIV-1 Env) and (ii) the 447-52D mAb (which recognizes a linear epitope in the V3 region of HIV-1 Env) (Table 2). We generated hybrid αIMs in which either the full BC or FG loop was replaced with a series of serine residues (equal in length to the wild-type BC or FG loop). These BC hybrids, FG hybrids, and parental αIMs were tested by a phage ELISA. Figure 6 shows the VRC-01 αIM hybrids were uniformly unable to bind to the VRC-01 mAb, unlike their respective parental αIMs. In contrast, one of the two 447-52D αIM hybrids retained the ability to bind efficiently to its cognate antibody. Figure 6 shows that a derivative of clone J2, containing a "Ser-replacement" BC loop, was able to bind to the 447-52D mAb. In contrast, serine-replacement of either the BC or FG loop in clone J3 abrogated binding to 447-52D, suggesting that both loops can contribute to binding of some αIMs to their cognate antibody.

To confirm this, we further characterized the VRC-01 3-2 αIM by using a variant form of combinatorial scanning mutagenesis. Specifically, we used a modified serine-scanning library to analyze the contribution of individual amino acid residues on the BC and FG loops to the interaction between anti-idiotypic monobodies and their cognate antibodies. Serine was used to replace wild-type residues because it is a small, hydrophilic amino acid that might be less disruptive to the overall structure of solvent-exposed loops than the more commonly used, but hydrophobic, alanine side chain.

To perform this analysis, we generated a "minilibrary" in which the amino acid residues in the BC and FG loops (i) were the wild-type sequence or (ii) were replaced with serine [except for methionine and histidine residues, which were replaced with a mixture of asparagine, arginine, and isoleucine (this was necessary because of the degeneracy of the genetic code)]. Phage prepared from this library were then separately selected for binding to the VRC-01 anti-Env mAb or to the irrelevant M2 anti-FLAG mAb. Table 3 shows the frequencies of the wild-type amino acids found in the selected clones. Sequence data for clones selected against the irrelevant M2 anti-FLAG mAb illustrate the diversity of the modified serine-scanning minilibrary. In contrast, sequences of clones selected for binding to the VRC-01 mAb demonstrate that the original sequence of the entire FG loop is important for VRC-01 mAb binding, along with four of seven residues in the BC loop. Thus, for the VRC-01 3-2 αIM, important contact residues were present in both the BC and FG loops.

Characterization of Anti-Idiotypic Monobodies That Are Reactive with mAbs 2F5 and 4E10. We also characterized αIMs that are reactive with mAbs that recognize linear antigenic epitopes. For this analysis, we chose 2F5 and 4E10 reactive αIMs that showed partial homology with the corresponding native mAb epitopes from HIV-1 gp41; we also selected a clone (4E10 5-2 αIM) that did not show homology to the 4E10 epitope. We assayed the ability of these αIMs, as well as an irrelevant αIM [F10λ (negative control)] and the full-length MPER peptide [ELLELDKWASLWNWFDITNW-LWYIK (positive control)], to bind to the 2F5 and 4E10 mAbs using a competition ELISA. Figure 7A shows that the 2F5-17 αIM was able to competitively inhibit the binding of the 2F5

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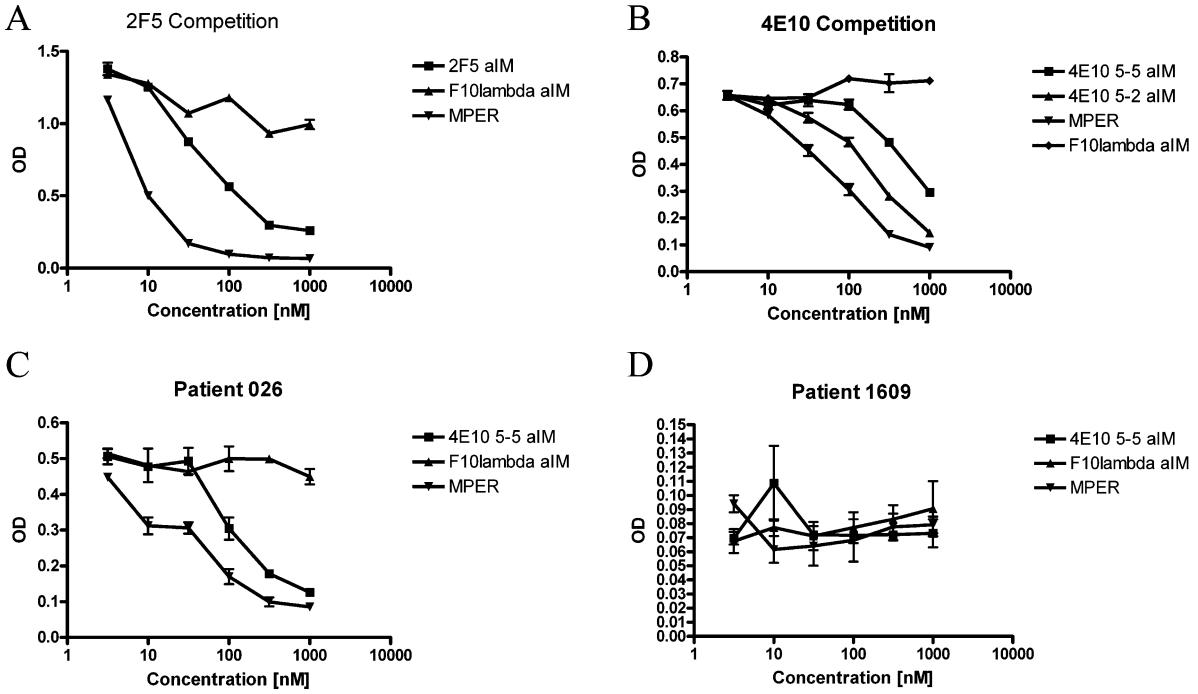

Figure 7. 2F5 and 4E10 αIMs competitively inhibit the binding of their cognate antibodies to their target epitopes in HIV-1 Env. (A) The 2F5 epitope peptide conjugated to BSA was immobilized in triplicate wells. A fixed concentration of 2F5 mAb was then preincubated with half-log dilutions of 2F5 αIM, MPER peptide, or an irrelevant αIM (F10λ) before addition to the coated plate. After the samples had been washed, bound 2F5 mAb was detected using the anti-human IgG−HRP conjugate. (B) The 4E10 epitope peptide conjugated to BSA was immobilized in triplicate wells. A fixed concentration of 4E10 was then preincubated with half-log dilutions of 4E10 αIM (5-5 or 5-2), MPER peptide, or an irrelevant αIM (F10λ) before addition to the coated plate. After the samples had been washed, bound 4E10 mAb was detected using the anti-human IgG−HRP conjugate. (C and D) Serum from an HIV-positive person (026) (C) or from an HIV-negative person (1609) (D) was preincubated with half-log dilutions of 4E10 5-2 αIM, 4E10 5-5 αIM, MPER peptide, or F10λ αIM before addition to a plate coated with the 4E10 epitope peptide. After the samples had been washed, bound 4E10 mAb was detected with the anti-human IgG−HRP conjugate.

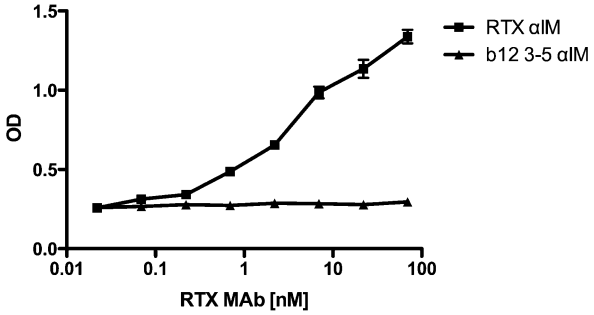

Figure 8. Rtx αIM can be used as a sensitive probe to detect Rituximab in normal human sera. Rtx αIM or b12 3-5 αIM was immobilized in wells in triplicate. Rituximab was then serially diluted in 1% pooled normal human sera (itself diluted in blocking solution) and added to the wells. After extensive washing, bound Rituximab was detected with the anti-human IgG−HRP conjugate.

mAb to its cognate peptide. Similar results were also obtained with both 4E10 αIMs (Figure 7B), including the αIM with no amino acid homology to the 4E10 peptide epitope (4E10 5-2 αIM). The results in Figure 7 also suggest that the affinity of the 2F5-17 αIM for its cognate antibody is ~10-fold lower than the affinity of the MPER peptide for the 2F5 mAb (Figure 7A). Similar results were obtained with the 4E10-specific αIMs, although the 4E10 5-2 protein appeared to bind its cognate antibody with only ~2−3-fold reduced affinity, compared to that of the MPER peptide (Figure 7B).

Finally, we performed a competition binding experiment using serum from an HIV-positive person with high levels of serologic reactivity to the MPER peptide. In this case, the 4E10 αIMs competitively inhibited binding of human IgG antibodies to the MPER peptide, whereas an irrelevant αIM had no effect (Figure 7C). In contrast, the αIMs had no effect on the background MPER binding activity of an HIV-negative control serum (Figure 7D).

Use of Anti-Idiotypic Monobodies To Detect a Therapeutic Monoclonal Antibody in Human Sera. To examine the utility of αIM as a tool for probing antibody specificities in human sera, we conducted an experiment in which the Rtx mAb was serially diluted in 1% pooled normal human sera and then allowed to react with Rtx- or b12 3-5 αIM-coated ELISA plates. This analysis revealed that the Rtx αIM was able to detect Rituximab concentrations of ≤1 nM (Figure 8). This is considerably more sensitive than a conventional peptide ELISA for measurement of Rituximab concentration in human serum,[41] suggesting that αIM proteins may have utility for pharmacokinetic studies of therapeutic monoclonal antibodies.

Determination of the b12 bNAb Binding Affinity of the b12 3-5 αIM. We determined the affinity of the b12 3-5 αIM using SPR with the recombinant b12 Fab as the analyte and immobilized bio-αIM as the ligand. The results are shown in Figure 9. The calculated K_D of 43 nM is in the range of affinities observed with similarly sized two-loop libraries subjected to phage display-mediated enrichment.[3] While this affinity is relatively strong, it likely could be significantly improved using off-rate selections[1] to derive tighter binders that might function as more effective epitope mimics.

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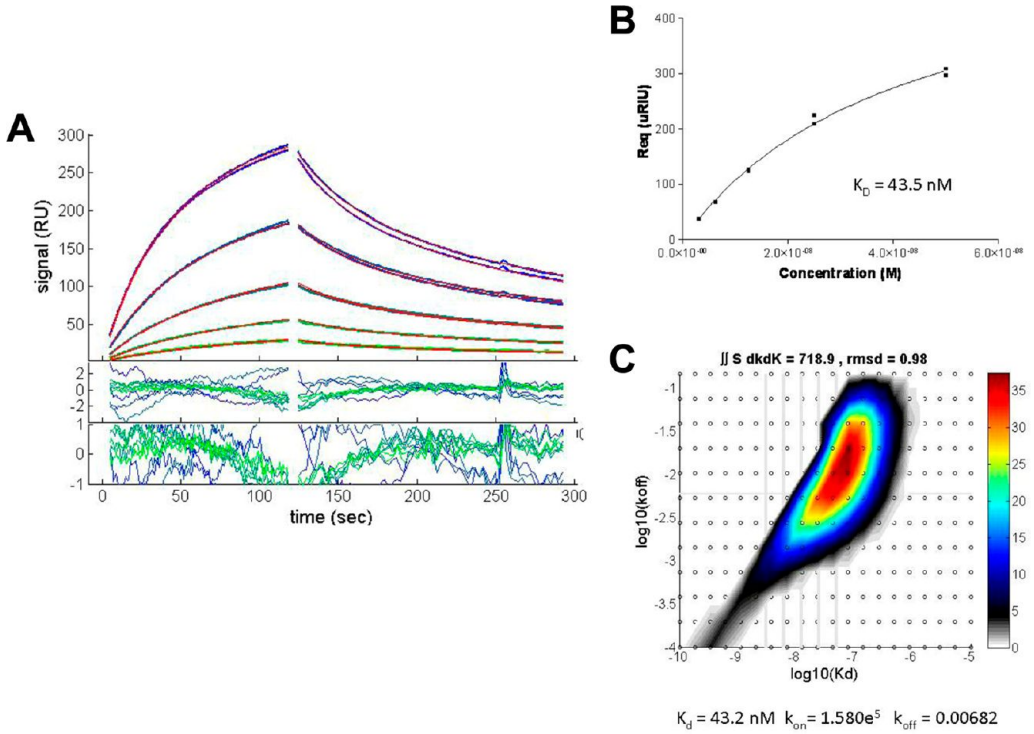

Figure 9. SPR analysis of binding of the b12 3-5 αIM to the b12 Fab. (A) Association and dissociation phases of binding of Fab to immobilized biotinylated b12 3-5 αIM captured on a streptavidin surface. Fab concentrations of 50, 25, 12.5, 6.25, and 3.125 nM were used (from top to bottom, respectively). (B) Langmuir isotherm of Fab binding. The K_D was determined by calculating the value of equilibrium R_{max} for each injection, plotted vs concentration and fit to a Langmuir binding isotherm {response = equilibrium response × $[(K_A[\text{b12 Fab}])/(K_A[\text{b12 Fab}] + 1)]$}. (C) Distribution analysis of the kinetics of binding of b12 Fab to surface-immobilized b12 3-5 αIM. Integration of the distribution inside a polygon drawn around the high-affinity peak gives a binding capacity of 718.9 RU, an average k_{off} of 0.0068 s^{-1}, and an average K_D of 43 nM.

DISCUSSION

In this study, we report the use of the 10th domain of human fibronectin (FNfn10) as a scaffold to generate anti-idiotypic monobodies recognized by a variety of monoclonal antibodies, including several HIV-1 Env-specific, broadly neutralizing antibodies (bNAbs). We successfully generated αIMs that mimic both linear (2F5 and 4E10) and conformational (b12) epitopes. Our original goal was to explore the use of a more compact, easily manipulated, and efficiently expressed display scaffold as an alternative to either peptide or antibody display to derive specific binding partners to antibodies. We believe such reagents could find use in a variety of applications such as protein purification, pharmacokinetic analysis of therapeutic antibodies, and, potentially, vaccine development.[3,4,47−49] We have recently described their potential utility for immune response profiling in autoimmune disease.[44]

We originally envisioned that two constrained loop domains on the FNfn10 scaffold protein would allow for complex, conformationally dependent, interactions with target antibodies. Our analysis of the mechanism of αIM and antibody interaction has revealed unexpected differences from this simple model. In the case of the two antibodies with complex discontinuous epitopes, b12 and VRC01, we observe very different modes of interaction. For b12, the 3-5 αIM exhibits a strong requirement at only two positions within the BC loop, with the remaining BC positions and most of the FG loop being rather tolerant of substitutions while retaining the ability to bind to b12. Remarkably, this αIM nevertheless accurately mimics the actual gp120 epitope with respect to three key residues of the VH domain required for antigen binding.

The VRC01 3-2 αIM represents nearly the complete opposite situation. In this case, almost the entire original sequence selected from the library must be maintained to retain binding to the mAb. The complete FG loop and four of seven residues within the BC loop were reselected in a combinatorial scanning of the two loops. Interestingly, the other VRC01-specific sequences selected have no similarity to each other. Presumably, these represent alternative conformations of the αIM lodged in the antibody paratope, each yielding a distinctive sequence. Further analysis using VRC01 CDR mutants will be necessary to fully define the mechanism of interaction.

For antibodies that recognize linear epitopes, exemplified by 4E10, 447-52D, and 2F5, Z13e1, some of the selected αIMs bore sequences in one of the loops that were homologous to the sequences of the reported linear epitopes. This suggests that these αIMs may be interacting with their cognate antibodies predominantly through a single loop. Other αIMs selected on these mAbs lack any obvious resemblance to the epitope and may interact in a manner that is more akin to the VRC01-specific αIMs. Despite the partial homology to the epitope found in the former type of αIMs, they were nevertheless found to compete with the identified linear peptide epitope for binding to the cognate antibodies and the 4E10 5-5 αIM could be used to detect serum antibodies in sera from an HIV patient with high reactivity to the linear MPER peptide.

Recent approaches to the generation of improved epitope mimics for HIV-1 bNAb include the grafting of both linear (2F5 and 4E10) and discontinuous (b12) epitope sequences onto computationally selected or remodeled protein scaffolds that optimize epitope display.[50−54] Our monobody-based approach offers a complementary method that can be readily

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combined with mutagenesis and selection techniques to isolate variants with altered specificity and affinity. This may facilitate the derivation of antigenic structures capable of reacting with germline precursors and/or early progenitors of somatically hypermutated bNAbs.[47−49,55] Other potential future applications may include the use of combinations of FNfn10-based αIMs and native epitopes grafted onto optimized scaffolds in heterologous prime-boost immunization regimens intended to promote immunofocusing on the targeted bNAb epitope.[52] Overall, the use of these robust, inexpensive, and easy to purify alternatives to conventional antigens could have broad applications in diagnostics, pharmacokinetic analyses of therapeutic monoclonal antibodies, and vaccine development.

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Notes

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ABBREVIATIONS

HIV-1, human immunodeficiency virus; αIM, anti-idiotypic monobody; Env, envelope surface glycoprotein of HIV-1; ELISA, enzyme-linked immunosorbant assay; bNAb, broadly neutralizing antibody; mAb, monoclonal antibody.

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