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Jordi Mata-Fink[a,f], **Barry Kriegsman**[a], **Yu Hui Xin**[a], **Hanna Zhu**[a], **Melissa Hanson**[c], **Darrell J. Irvine**[b,c,d,e,f], and **K. Dane Wittrup**[a,b,d,*]

[a]Department of Chemical Engineering, Massachusetts Institute of Technology (MIT), Cambridge MA, 02139, USA.

[b]Department of Biological Engineering, MIT, Cambridge MA, 02139, USA.

[c]Department of Materials Science and Engineering, MIT, Cambridge MA, 02139, USA.

[d]David Koch Institute for Integrative Cancer Research, MIT, Cambridge MA, 02139, USA.

[e]Howard Hughes Medical Institute, Chevy Chase, Maryland, 20815, USA.

[f]Ragon Institute of MGH, MIT and Harvard, Boston, Massachusetts, 02129, USA.

Abstract

gp120 is a substrate for protein engineering both for HIV immunogen design and as a bait for isolating anti-HIV antibodies from patient samples. In this work we describe the display of a stripped core gp120 on the yeast cell surface. Validation against a panel of neutralizing antibodies confirms that yeast-displayed gp120 presents the CD4 binding site in the correct conformation. We map the epitope of the broadly neutralizing anti-gp120 antibody VRC01 using both a random mutagenesis library and a defined mutant panel, and find the resultant epitope maps are consistent with one another and with the crystallographically identified contact residues. Mapping the VRC01-competitive antibodies b12 and b13 reveals energetic differences in their epitopes that are not obvious from existing crystal structures. These data suggest mutation sets that abrogate binding to broadly neutralizing antibodies with greater specificity than the canonical mutation D368R, useful in rapidly assessing the nature of a vaccine response.

Introduction

Glycoprotein gp120 of the envelope spike of human immunodeficiency virus (HIV) is the target of most anti-HIV antibodies generated upon infection or vaccination[1,2]. HIV mutates rapidly and gp120 tolerates tremendous sequence variability, rendering the vast majority of these antibodies ineffective, allowing the virus to evade immune recognition[3]. Certain regions of gp120, such as the docking site of CD4, must be conserved for the virus to retain fitness[4]. Antibodies that target these conserved epitopes have been shown to bind to and block virus regardless of clade, thereby negating the virus' main advantage[1]. Much of the focus of current HIV vaccine research involves the isolation of new neutralizing antibodies,

*Corresponding author wittrup@mit.edu MIT 76-261D, Cambridge MA, 02139, USA. Tel: 617-253-4578 Fax: 617-452-3293 .

understanding their structure/function relationships, and designing immunogens intended to elicit such antibodies by vaccination.

Advances in high-throughput screening techniques and new patient cohorts have led to an explosion in the discovery of broadly neutralizing antibodies. Whereas five years ago there were just a handful of known gp120-specific neutralizing antibodies—b12 against the CD4 binding site[5,6], 2G12 against a conserved glycan motif[7], and 17b and X5 against the chemokine co-receptor binding site[8-10]—there are now dozens on the rapidly-growing list[11-14]. Beyond just finding new neutralizing antibodies, analysis of patient antisera has identified common neutralization-sensitive epitopes[15-18]; crystal structures provide insights into the relationship between the structure of antibody-gp120 complexes and their function[19-21]; deep sequencing has revealed the likely somatic hypermutation pathway by which these antibodies evolved from the germ line[22,23]; and hypotheses about how these antibodies compensate for monovalent binding to sparse trimeric spikes on the viral surface have been tested[24,25].

VRC01 is the gold standard antibody against the CD4 binding site, because it is one of the broadest and most potent neutralizing antibodies discovered to date[26]. It was isolated from a patient sample using a hybrid HIV/SIV gp120 that had been "resurfaced" to remove all HIV epitopes except for the CD4 binding site. Similar screens with modified gp120 molecules that exhibit differential binding is how most neutralizing antibodies are identified and characterized[27-29].

Modifications are also made to gp120 when designing an immunogen to elicit neutralizing antibodies by vaccination[30]. Deliberate amino acid substitutions can, for example, lock the flexible molecule into an "open" conformation and overcome the considerable entropic penalty incurred upon binding[31-33]. Mutations can also disrupt antibody binding to irrelevant surfaces and encode glycosylation sites to shield undesired epitopes[34-36]. Immunodominant loops that distract from neutralizing epitopes can be removed entirely[37,38].

Trimeric gp160 is an alternative to monomeric gp120 for many of these applications. It has been shown that the monomer presents epitopes that are sterically inaccessible on the native trimer, and the immune system elicits non-neutralizing antibodies to these monomer-specific epitopes[19]. Additionally, some neutralizing antibodies recognize quaternary epitopes and thus cannot be faithfully captured by monomeric gp120[14]. Soluble trimers have been designed and used in in vitro screens and in vivo immunizations[39,40]. Engineered viruses are also used to present gp120 in its native conformation[15], but this requires additional safety measures that are not amenable to every lab.

Most of the previously-described gp120 variants are made by rational design, sometimes guided by computation, then individually constructed, secreted, and tested for binding by ELISA[35,41,42]. Yeast surface display provides an alternative, simple and flexible method for engineering complex glycoproteins[43,44]. Surface displayed proteins can be easily modified by random or rational mutagenesis, and binding phenotypes assayed by flow cytometry. We and others have engineered complex glycoprotein receptor ectodomains[45,46], and validated yeast surface display for fine resolution mapping of conformational epitopes[47].

Despite its utility, yeast display has not yet been used to engineer gp120 for immunogens or as bait for isolating neutralizing antibodies. In this work, we report the display of gp120 on yeast, characterize its binding to a panel of broadly neutralizing antibodies and map the epitopes of several of these antibodies, demonstrating the potential for yeast display to accelerate immunogen design.

Results

Display of gp120 on yeast. The gene for gp120 from HIV strain YU2[48] was subcloned into a yeast display vector with a C-terminal Aga2p fusion partner (FIG 1A). Yeast displaying the full protein did not bind to antibody b12 (data not shown), so extensive modifications were made to strip the protein of flexible loops that might misfold or cause steric occlusion of the b12 epitope (FIG 1A and Supplementary Table 1). These modifications are similar, though not identical, to the original core gp120 crystalized in complex with CD4[49]. The flexible gp41-interactive regions at the N- and C-termini (aa 1-89, 493-511)[50] were excised entirely.The long hyper-variable loops[3,51]—V1/V2 (aa 124-198), V3 (aa 298-329), and the bridging sheet   20/21 (aa 422-437)—were replaced with the short, glycosylated amino acid linkers GNGS, GNGSG, and GGNGS respectively. An additional set of mutations (Q114N, L116T) introduced an N-linked glycosylation sequence at a site previously identified as important for masking an epitope on the V1/V2 stem[35].

Despite thesemodifications, the resultant "stripped core" gp120 retains the structurally rigid outer domain that has been extensively studied elsewhere[4,52,53].The protein is expressed well on the yeast surface (1×10^5 copies per cell; standard for yeast surface display) and conserves the structure of the CD4 binding site as measured by binding to CD4 and to a panel of neutralizing antibodies (FIG 1B, 1C).

Broadly neutralizing anti-gp120 antibodies VRC01, PGV04 (isolated from the same patient as VRC01 but by a different protocol)[26], and b12[5,6], as well as the non-neutralizing CD4-directed antibodies b6 and b13[54] were titrated on yeast displaying stripped core gp120 and the binding measured by flow cytometry. The equilibrium binding constants ranged from 24 pM for b6 to 0.9 nM for PGV04 and b13. Yeast-displayed stripped core gp120 also bound CD4-Fc with a binding constant of 5.6 nM, despite the removal of the bridging sheet that comprises a substantial portion of the CD4 binding site. These binding data are consistent with the single-digit nM affinities measured for CD4 binding site-directed antibodies to several gp120 constructs by other methods[26], and with binding of multivalent CD4 constructs to both "liganded" and "unliganded" conformations of gp120 [20].

Other antibodies of interest in the field of HIV vaccine design were tested as well, but did not bind to the stripped core construct (data not shown). Antibodies 17b and X5 that recognize the chemokine receptor epitope on gp120[8-10]—called the CD4-induced or CD4i epitope because it is only induced upon CD4 engagement—did not bind, which is expected given that much of the CD4i epitope was removed when the bridging sheet was replaced with a short linker. Non-neutralizing antibody F105[55], whose crystal structure (PDB ID: 3HI1) indicates that it too makes significant contacts with the bridging sheet[19], did not bind.Antibodies PGT-121 and PGT-128, known to recognize an epitope on variable loop V3[11,12], did not bind to the V3-less construct. Antibody 2G12, which recognizes an array ofhigh-mannose glycans on the surface of gp120[56-58]also did not bind the stripped core displayed on yeast.*S. cerevisiae* hyper-mannosylates*N*-linked glycans and produces glycoforms different from those recognized by 2G12[59].Others have engineered *S. cerevisiae*such that its glycoproteins are recognized by 2G12[60]; display of stripped core on this modified yeast strain may introduce 2G12 sensitivity.

To ensure that the binding to CD4 binding site-directed antibodies was not an artifact of yeast display, we secreted stripped core gp120 from HEK 293 cells both as a monomer and as an Fc-fusion, and measured its binding to the single-chain variable fragment (scFv) of VRC01 displayed on yeast. The equilibrium dissociation was found to be 8.7 nM for monomeric stripped core and 1.1 nM for the dimeric Fc-fusion (FIG 1D), consistent with previous titrations where the arrangement of gp120 and antibody was inverted.

Mapping the VRC01 epitope with a random mutagenesis library.The broadly neutralizing antibody VRC01 was used as a test case for epitope mapping with yeast-displayed stripped core gp120. The crystal structure of the antibody in complex with gp120 from HIV strain 93TH057 has been solved (PDB ID: 3NGB)[20] and can be used to validate mapping results. The antibody is one of the broadest and most potent neutralizers of HIV yet discovered, and a deeper understanding of its function many help inform immunogen design and guide the search for new neutralizing antibodies.

We first mapped the VRC01 epitope using a library of randomly mutagenized gp120 molecules, as described previously[47]. The library was generated by PCR with an error-prone polymerase under conditions of low mutagenesis. Its diversity was 7×10^7 members, sufficient to sample all possible single amino acid substitutions at each of 285 positions. Yeast displaying the gp120 library were incubated with 50 nM VRC01 and sorted for loss of binding to the antibody. 0.6% of the original library was collected in this first sort, and a second round at the same antibody concentration further enriched the population (FIG 2A, 2B).

A third round of sorting was performed with a conformational control antibody to exclude allosteric mutations that ablate VRC01 binding from afar without making direct contact with the antibody. gp120-specific antiserum from a mouse immunized with the stripped core construct was used as a conformational control in this third sort.When the yeast library was incubated with antiserum, only 5.5% of cells retained binding and were collected (FIG 2C). This suggests that the majority of clones isolated in the VRC01 sorting rounds contained allosteric mutations, and underscores the importance of a conformational control antibody.

To ensure that the antiserum in fact recognized conformational epitopes on the protein, yeast displaying stripped core were first heated in the presence of 8 M urea to denature the protein before binding was assayed. Antiserum bound the denatured stripped core more weakly than it did the native protein (FIG 2D), suggesting that it recognized some conformational epitopes on the protein. To ensure that the loss of binding was not due to fewer total proteins present on the yeast cell surface, the binding signal was scaled by display signal from the CMyc epitope tag.

Thirty-two clones from the library were sequenced after the third sort. Mutationsthat introduced or replaced a cysteine or proline were removed from the analysis, as were positions that were mutated only once. The remaining 21 mutations occurred at five positions: 280, 368, 456, 458, and 469 in standard HxB2 numbering[61]. These positions fall within the VRC01 footprint as defined by the antibody contact residues in crystal structure 3NGB (FIG 2E). Of the nine single mutations that were discarded from the analysis, one (N278Y) resides in the VRC01 epitope and seven came from clones that also contained either a VRC01 epitope mutation or a change to/from cysteine/proline.

The random mutagenesis epitope map is consistent with the structural epitope, but the method requires access to a cell sorter and repeated rounds of sorting, sequencing and analysis. To enable higher-throughput analysis of antibodies and antisera, we constructed a small mutant library useful for rapid mapping of gp120-specific antibodies simply by flow cytometry, without sorting, sequencing, or the need for a validated conformational control antibody.

Making a defined mutant panel. We generated a homology model of stripped core gp120 with the protein structure modeling program MODELLER[62], and 31 evenly-spaced surface residues were chosen for mutagenesis. Amino acid substitutions were selected to be highly disruptive based on differences in polarity, charge, and size. To guard against allosteric mutations, only amino acids present with at least 0.1% frequency in the Los Alamos

National Laboratory HIV Sequence Database were considered at each position. Thus, prevalence in nature was used as a proxy for properly-folded gp120. The only exception to this rule is the mutation D368R—a known CD4 binding site disruptor commonly used to screen for CD4-directed antibodies from patient samples[63]—which is included in the library though absent from the database. The location and identities of selected amino acid substitutions are shown in FIG 3A, 3B and Table 1.

Mapping the VRC01 epitope with the mutant panel. The epitope of VRC01 was mapped with the defined mutant panel. VRC01 was incubated with the library at 300 pM, and binding signal analyzed using a plate-reader cytometer. Six mutations were found to disrupt binding to VRC01: the expected D368R, as well as S365K, T455E, G459E, D461I, and G473R (FIG 3C). These residues are consistent with the map from the random mutagenesis library, and nicely overlay the antibody footprint as defined by crystal structure 3NGB (FIG 3B). The positions identified by the epitope map fall along the ridge comprised of the CD4 binding loop, variable loop V5, and beta strand 24, all known to be energetically important for VRC01 binding[64]. Two mutations in loop D, T278K and K282V, were found not to disrupt VRC01 binding to stripped core despite the fact that the loop appears to make contact with VRC01 in the native trimer[20].

An important consideration is that the quality of the map generated with this panel is sensitive to the antibody concentration in the assay (FIG 3D). When the library was incubated with VRC01 at 30 nM, only D368R showed any appreciable disruption of binding. Incubations at 0.3 and 3 nM resulted in a clear distinction between the six mutations listed above and all others. At lower VRC01 concentrations, this difference diminished as the overall binding signal decreased across the board. Fitting binding isotherms to the mapping data for each mutant illustrates this phenomenon (FIG 4A, Supplementary Table 2). Non-epitope mutants have equilibrium binding constants between 0.1-1 nM, similar to that of wild-type stripped core gp120. By contrast, the K_D for D368R was greater than 100 nM, which explains why the mutant fails to bind at even the highest tested concentrations of VRC01. A cluster of weaker mutations— the same S365K, T455E, G459E, D461I, and G473R identified above—have a K_D an order of magnitude higher than wild-type gp120. There is a narrower window of antibody concentration for identifying these five mutations in our screen.

Mapping other anti-gp120 antibodies. Two other important CD4-directed anti-gp120 antibodies, b12 and b13, were then mapped with the mutant panel. Antibody b12 is neither as broadly cross-reactive nor as potent as VRC01 but for many years was the gold standard of HIV neutralization. Antibody b13 also binds the CD4 binding site, but its offset footprintrenders it incapable of binding trimeric gp120 envelope on HIV[19]and thus incapable of neutralizing the virus.

Antibodies b12 and b13 were mapped over a range of concentrations near their K_D (FIG 4B and 4C, Table 2, and Supplementary Table 2). Mutations D368R, R419G, and T455E strongly disrupt binding to b12, while mutations K282V, S365K, and I467K have weaker effects. For b13, D368R is strongly disruptive and R419G is marginally so. When these mutations are mapped onto the homology model, they are consistent with the contact residues from the corresponding crystal structures (2ND7 and 3IDY, respectively) (FIG 4E, 4F).

The residues identified by epitope mapping by yeast surface display are also consistent with those identified by previous mapping efforts reported in the literature. Amino acid substitutions at positions 368 and 457 were found to disrupt binding of CD4 to gp120[65]. Alanine scanning identified residues 365, 455, 458, and 473 as important for b12 binding[66],

and residues 474-476 for binding of CD4 binding site-reactive antibodies cloned from infected individuals[67]. Computational analysis of envelope sequences found that conservation at positions 364, 369 and 461 were indicative of b12 neutralization[68].

Identification of combination mutants that bind differentially to VRC01, b12, and b13. D368R ablates binding to all three CD4 binding site-directed antibodies tested, despite variation in their epitopes and important phenotypic differences. The fine resolution conformational epitope maps suggest that some of the weaker mutations may be more specific disruptors than D368R, but they are very sensitive to antibody concentration. We decided to combine the weaker mutations to see if they can act cooperatively to knock out binding over a broader antibody concentration range while maintaining specificity.

Six constructs with either two or three mutations in combination were made by site-directed mutagenesis and displayed on the surface of yeast (Table 3). The constructs were first titrated against gp120-specific mouse antiserum (FIG 5A). For all constructs, the binding thermodynamics were similar to that of D368R, suggesting that the multiple mutations do not grossly disturb the overall protein structure.

The constructs were then titrated against antibodies VRC01, b12, and b13 (FIG 5B, 5C, 5D). Multi-mutation construct MM3 (S365K, T455E, G473R) knocked out binding to VRC01 and b12 to a greater than or equal extent as canonical mutation D368R, but had no effect on binding of b13. This construct may be useful for screening out b13-like antibodies that approach the CD4 epitope at an angle not conducive to neutralization. Multi-mutation construct MM5 (T459E, G473R) is even more specific for VRC01. It abrogates binding to VRC01 more completely than D368R, but has negligible effects on both b12 and b13 binding. This construct may be useful for identifying precisely VRC01-like antibodies from patient samples or immunization antisera.

Discussion

There is much effort in the HIV vaccine field to modify gp120 to make subunit vaccine immunogens, and as bait to isolate and characterize antibodies from patient samples. Yeast surface display is a simple and powerful tool for engineering complex glycoproteins[43,44,46], and random mutagenesis yeast-displayed libraries have been used for conformational epitope mapping of EGFR [47], West Nile virus[69], and influenza hemagglutinin[70] previously. In this work we demonstrate the display of a stripped core gp120 on yeast, and show by binding to a panel of neutralizing antibodies that the vulnerable CD4 binding site epitope is wellpresented. This platform enables rapid screening of candidate immunogens and high-throughput analysis of serum samples.

As an example of the potential of yeast surface display, we have mapped the epitope of the broadly neutralizing antibody VRC01 using a random mutagenesis library screened by FACS. This is a convenient and established way to generate fine resolution maps of conformational epitopes[47,71-73]. In the method, a random library of gp120 variants with single point mutations is displayed on the surface of yeast and sorted for loss of binding to the antibody. Mutants that disrupt binding likely form part of the epitope. We isolated mutations at five positions—280, 368, 456, 458, and 469—that are consistent with the known contact area of VRC01 from crystallography.

Because it can be difficult to distinguish mutations in the epitope from allosteric mutations that disrupt the binding site at a distance, the random library method requires a conformational control antibody that binds non-competitively elsewhere on the protein and can report whether the molecule is correctly folded. In this work we relied upon gp120-specific mouse antiserum collected in-house to eliminate mutations that allosterically disrupt

VRC01 binding. The small fraction of the library (5.5%) that retained binding to serum after having been sorted for loss of binding to VRC01 underscores how critical the conformational control is.

A small mutant panel such as we constructed in this work has both advantages and disadvantages relative to the random library screening approach described previously[47]. The initial effort to identify a set of ~30 mutants is balanced against the relative speed with which a given antibody can then be tested. A defined library of rationallychosen mutations, though requiring more work to make, can be screened and analyzed without any DNA sequencing, giving results in just a few hours.

VRC01 was mapped with the mutant panel, and the results were consistent with both the random mutagenesis mapping and crystallography (FIG 4D vs. FIG 2E). It is important to note that the quality of the map from the defined mutant panel is sensitive to the antibody concentration in the assay (FIG 3D). This sensitivity is less evident with random library mapping because only strongly disrupting mutations are isolated by that method. By contrast, each mutant in the panel, although chosen to be disruptive, will have a particular equilibrium binding constant. At too high a concentration all the isotherms are saturated and will show similar binding signals. At too low a concentration, noise will make it impossible to distinguish strong from weak binders.

Two other anti-gp120 antibodies, b12 and b13, were mapped with the mutant panel, and the results agreed with crystallography. Crystal structures give accurate and detailed spatial data; epitope mapping can provide additional information about epitope energetics. The three mapped antibodies all bind the CD4 binding site but have significantly different neutralization phenotypes. VRC01 is broadly neutralizing; b12 is weakly neutralizing; and b13 is non-neutralizing. Our epitope map highlights the different energetically important residues in the epitopes of these three antibodies.

One shared mutation among the three epitope maps is D368R, the canonical CD4 knockout mutation. This mutation is widely used in the HIV vaccine field to test the specificity of antibodies elicited by immunization and to screen patient samples for new neutralizing antibodies[15,17,26-28]. Our mapping titrations indicate why this mutation is so favored. It abrogates binding to all CD4-directed antibodies, and shifts the K_D to such a degree that the effect is observed regardless of the antibody concentration. This is particularly useful when screening polyclonal antisera in which the precise concentration of individual antibodies is unknown.

But the fact that D368R is common to all three epitope maps is also a detriment to its use, because the mutation cannot distinguish between antibodies with similar epitopes but significantly different phenotypes. Our mapping data suggests that combinations of some of the weaker mutations may have equal potency but be more specific disruptors than D368R. The multiple mutation construct MM3 (S365K, T455E, G473R) knocks out binding only to VRC01 and b12, but not b13. The construct MM5 (T459E, G473R) abrogates binding to VRC01 alone. These constructs may be useful for identifying precisely VRC01-like antibodies from patient samples or immunization antisera. No binding assay can replace a neutralization assay to determine antibody function, but improved specificity can help improve the initial screen, particularly in cases where there may be limiting quantities of serum (e.g. mouse immunization).

Methods

Display of gp120 on yeast. The gene for gp120 from HIV strain YU2[48] (Genbank Accession No. M93258) was subcloned into the yeast display vector pCHA with a C-terminal Aga-2

fusion partner with two epitope tags, CMyc (EQKLISEEDL) and HA (YPYDVPDYA). The protein was displayed on Saccharomyces cerevisiae strain EBY100 using a standard surface display protocol[74]. In brief, EBY100 yeast were transformed with plasmid (Frozen-EZ Yeast Transformation II Kit, Zymo Research, Irvine, CA), grown to mid-log phase in SD-CAA media at 30°C, and induced in galactose-containing SG-CAA media for 24 hr at 20°C. Approximately 1×10^5 gp120 molecules are displayed per yeast, as measured by quantitative flow cytometry (data not shown).

Modifications to gp120. Antibody staining of epitope tags indicated that the full-length gp120 was displayed on the surface of yeast, but no binding was observed to the antibody b12 (data not shown). Extensive modifications were made to remove flexible loops that might occlude the CD4 binding site or cause the protein to misfold. To this end, we removed the flexible gp41-interactive region at the N- and C-termini (aa 1-89, 493-511)[50] and replaced the hyper-variable loops V1/V2 (aa 124-198), V3 (aa 298-329), and the bridging sheet 20/21 (aa 422-437) with the short, glycosylated amino acid linkers, GNGS, GNGSG, and GGNGS respectively. An additional double mutation (Q114N, L116T) introduced an N-linked glycosylation sequence at a site previously identified as important for masking an epitope on the V1/V2 stem[35]. All amino acid numbering is based on the HxB2 numbering convention[61]. The full sequence is listed in Supplementary Table 1.

Secretion of gp120 from HEK 293 cells. The stripped core gp120 was subcloned into a mammalian expression vector based on gWiz (Genlantis, San Diego, CA), either by itself or downstream of the Fc domain of mouse IgG2a. All constructs also have a his6 tag. A suspension culture of HEK 293 cells grown in serum-free medium (Freestyle 293, Life Technologies, Carlsbad, CA) was transfected with DNA using PEI as per manufacturers instructions. Supernatant was harvested after one week and purified on Talon metal affinity chromatography resin (Clontech, Mountain View, CA) followed by size-exclusion chromatography.

Making the random mutagenesis library. A random mutagenesis library was generated by PCR with Mutazyme II error-prone polymerase (GeneMorph II Kit, Agilent, Santa Clara, CA). To ensure a low mutagenesis rate, 400 ng target DNA was amplified for 24 cycles with flanking primers. The re-amplified PCR product was combined with digested pCHA vector and electroporated into EBY100 yeast, where the full plasmid was reassembled by homologous recombination[74]. The final library size was estimated to be 7×10^7.

Homology modeling with MODELLER. The protein structure modeling program MODELLER[62] (http://salilab.org/modeller) was used to generate a homology model of the yeast-displayed stripped core YU2 gp120. Though several crystal structures of gp120 exist in the Protein Data Bank, none is an exact analog for the yeast-displayed molecule. We used the crystal structure of gp120 bound by VRC01 (PDB ID: 3NGB, strain 93TH057) as a structural template, though basing the model on alternate gp120 templates did not alter the model significantly (Supplementary Figure 1).

Algorithm for selecting surface residues and amino acid substitutions for the mutant panel. The homology model of stripped core gp120 was rendered in PyMOL (PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC.) and 31 evenly-spaced sites with large solvent-exposed surface areas were selected for mutagenesis. Amino acid substitutions were chosen to be highly disruptive of binding, yet not so disruptive as to misfold the protein. We evaluated each amino acid substitution on three biophysical axes: polarity (hydophobic, neutral, or polar), charge (negative, neutral, or positive), and size (greater than or less than 160 Å^2 surface area)[75-77]. A value of +1 was assigned for every unit step on each axis, and the total disruption score was calculated by summing the net differences

between two amino acids. Higher scores correspond to more disruptive changes. For example, a mutation from glutamic acid to tyrosine would be scored +3 total: +2 for the change from polar to hydrophobic, +1 for the change from negative charge to neutral, and +0 for size because both surface areas are greater than 160 Å^2.

To guard against allosteric mutations, we excluded all amino acid substitutions that are present at less than 0.1% frequency in the Los Alamos National Laboratory HIV Sequence Database (http://hiv.lanl.gov). An extremely disruptive mutation that that destroys the fold of gp120 would likely ablate viral fitness too. The only exception to this rule is the mutation D368R, a frequently used CD4 binding site disruptor, that is included in the library though absent from the database.

Substitutions were made by site-directed mutagenesis. The mutant panel was limited to 31 clones and one wild-type stripped core gp120 so that three mappings could be performed on a single 96-well plate on a high-throughput flow cytometer. A library with more members would provide a finer resolution map at the cost of overall assay throughput.

Flow cytometry. Anti-gp120 antibodies were either produced from HEK cells or a generous gift from Dennis Burton. CD4-Fc was purchased from Sino Biological (Beijing, China). Yeast cells displaying protein were incubated with anti-gp120 antibodies and anti-epitope tag antibodies, either mouse anti-HA (clone 16B12, Covance Inc., Emeryville, CA) or chicken anti-CMyc (Gallus Immunotech, Fergus, Ontario). Secondary labeling was performed with goat anti-mouse, -human, or -chicken antibodies conjugated to Alexa Fluor dyes (Life Technologies, Carlsbad, CA) at recommended dilutions. Individual samples were analyzed on an Accuri C6 cytometer (BD Accuri Cytometers, Ann Arbor, MI); 96-well plates were run on a FACSCalibur HTS with a high-throughput plate sampler (Becton Dickinson, Franklin Lakes, NJ); sorting was performed on a MoFlo instrument (Beckman Coulter, Brea, CA).

gp120-specific mouse serum. Stripped core gp120 was secreted from HEK 293 cells as a fusion to mouse IgG2a Fc, and purified by Talon affinity chromatography. The protein was mixed with AddaVax (Invivogen, San Diego, CA) an oil-in-water emulsion adjuvant similar to MF59 licensed for use in Europe. BALB/c mice (Charles River Laboratories, Wilmington, MA) were immunized with 7.5 pmol gp120 subcutaneously at the base of the tail, and boosted again six weeks later. Antisera were titrated on gp120-displaying yeast (Figure 2D). A dilution of 1:200 saturated the yeast-displayed gp120, and was used as the working dilution for antisera in all experiments in this report. To demonstrate conformational specificity of the antisera, titrations were performed on yeast displaying gp120 that had been incubated with 8 M urea and heated for 30 minutes at 82C to denature the protein. In this assay, binding MFU were scaled by display MFU (CMyc epitope tag) to ensure that the loss of binding signal was not due to fewer total proteins present on the yeast cell surface.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Engineered gp120 are used as immunogens and as bait for isolating anti-HIV antibodies

- Stripped core gp120 presenting the CD4 epitope is displayed on the yeast cell surface

- Rapid conformational epitope mapping of antibody VRC01 is performed by two methods

- Mapping of b12 and b13 captures subtle energetic differences between similar epitopes

- Maps suggest mutations that may better identify and assess neutralizing antibodies
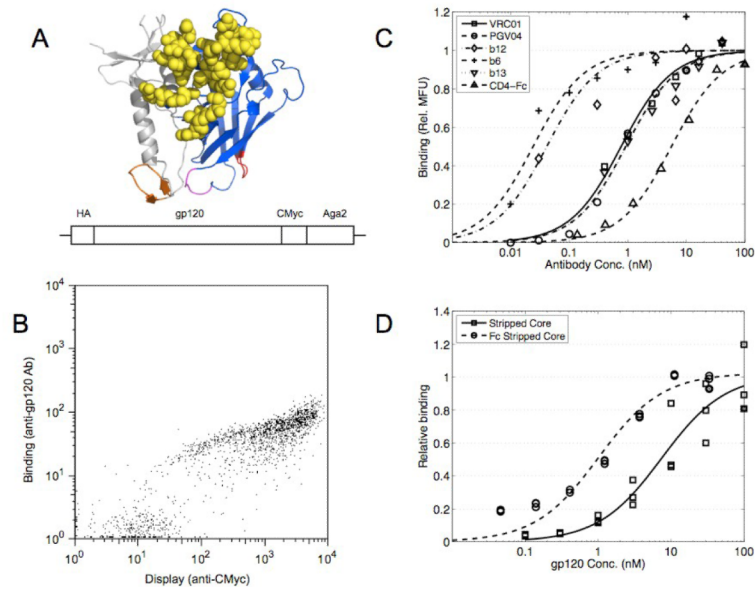
Figure 1.
Yeast display of gp120. (a) Homology model of stripped core showing the inner domain (white), outer domain (blue), the short glycosylated peptides that have replaced the V1/V2 loop (orange), V3 loop (red), and bridging sheet (purple), and the VRC01 contact residues (yellow). Beneath it, a schematic of yeast display vector pCHA showing gp120, the C-terminal Aga2 fusion partner, and two epitope tags. (b) Representative flow cytometry dot-plot of yeast displaying stripped core gp120, bound to both an anti-CMyc antibody (x axis) and the broadly neutralizing antibody VRC01 (y axis). (c) Binding isotherms of yeast-displayed stripped core gp120 to a panel of CD4 binding site-directed antibodies. Equilibrium K_D values for the antibodies are as follows: VRC01, 0.8 nM; PGV04, 0.9 nM; b12, 41 pM; b6, 24 pM; b13, 0.9 nM; CD4-Fc, 5.6 nM. (d) Binding of secreted stripped core gp120 to yeast displaying the scFv of antibody VRC01. Equilibrium K_D values for the interactions are: Stripped Core, 8.7 nM; Fc Stripped Core, 1.1 nM.
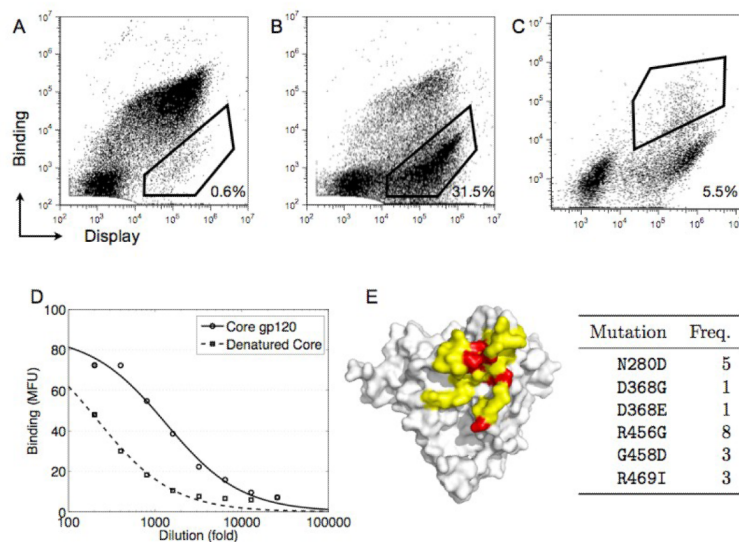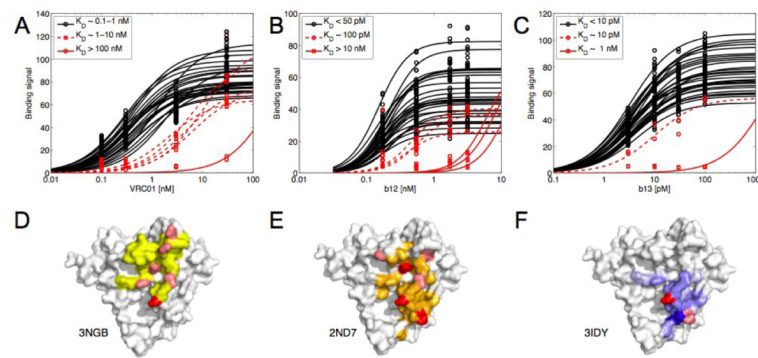
Figure 2.
Epitope mapping of VRC01 with a random mutagenesis library. (a-c) Sequential FACS sorting plots of the library incubated with (a) 50 nM VRC01, (b) 50 nM VRC01, and (c) a 1:200 dilution of anti-gp120 antiserum. The fraction of total cells collected is shown on each plot. (d) Titration of serum from an immunized mouse on yeast displaying gp120 (solid) and yeast displaying gp120 whose structure has been disrupted by incubation with 8 M urea (dashed).(e) Mutated residues from 32 sorted clones highlighted on the homology model of stripped core gp120. Residues in red are the sequenced mutations; residues in yellow are the contact region of VRC01 as determined from crystal structure 3NGB. The accompanying table lists the mutations and their frequency.
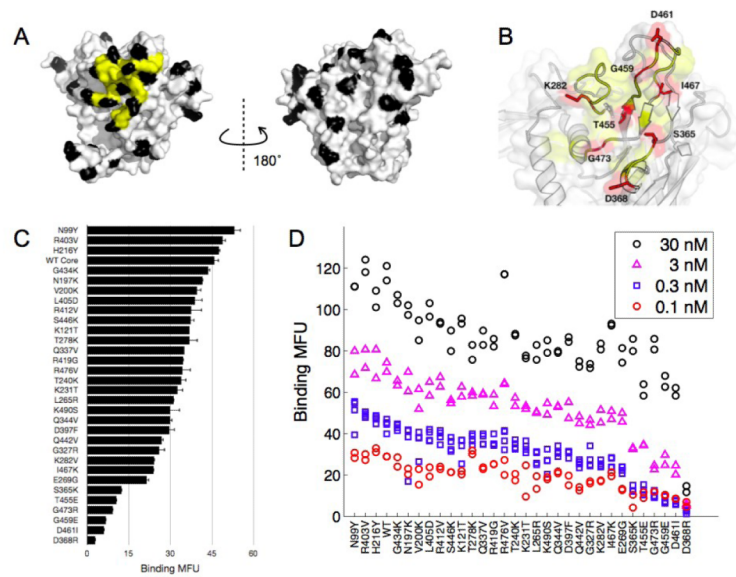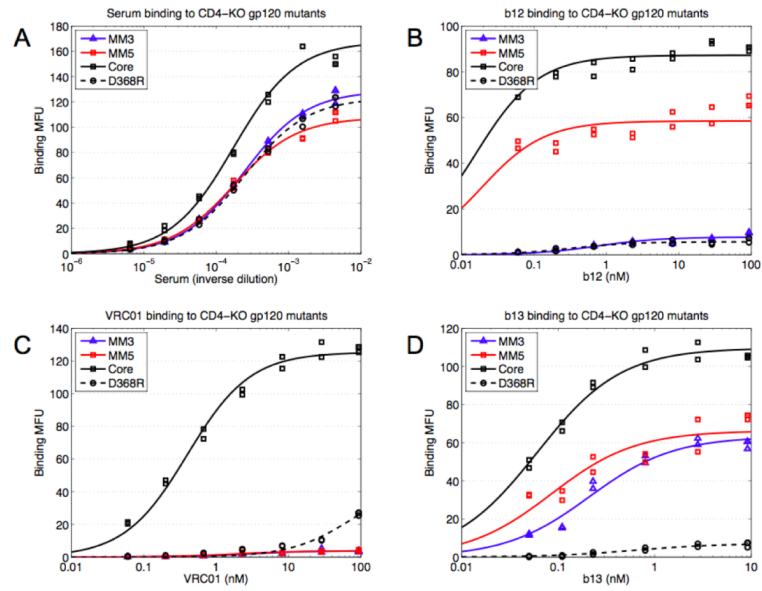
Figure 3.
Epitope mapping of VRC01 with a defined mutant panel. (a) Homology model of stripped core gp120 with mutant panel residues shown in black. The contact surface of VRC01 from crystal structure 3NGB is shown in yellow. (b) Close-up of the VRC01 epitope with select residues in the mutant panel labeled and shown in red. (c) Binding of 300 pM VRC01 to the mutant panel as measured by flow cytometry. Mutations are arranged in decreasing order of binding MFU. (d) VRC01 binding to the mutant panel over a range of VRC01 concentrations. At the highest concentration (30 nM) only D368R disrupts binding. At the lowest concentration (100 pM) it is difficult to distinguish differences in binding signal. At intermediate concentrations, an epitope is well defined by loss of binding to: S365K, T455E, G473R, G459E, D461I, and D368R.

Figure 4.
Epitope mapping of CD4 binding site-directed antibodies. (a-c) Binding of antibodies VRC01 (a), b12 (b), and b13 (c) to the defined mutant panel at a range of concentrations. Monovalent equilibrium binding isotherms are fit to the data. Mutations that do not significantly change the binding thermodynamics are shown in black. Those that weakly disrupt binding are shown in red with dashed lines. Mutations that strongly disrupt binding are drawn in solid red lines. Mutations are listed in Table 2. (d-f) Mutations that affect binding to VRC01 (d), b12 (e), and b13 (f) are shown on the homology model of stripped core gp120. Mutations that weakly disrupt binding are colored pink; those that strongly disrupt binding are colored red. The contact residues of each antibody as defined by crystallography are shown in yellow (VRC01, PDB ID 3NGB), orange (b12, PDB ID 2ND7), and blue (b13, PDB ID 3IDY) respectively.

Figure 5.
inding of anti-gp120 antibodies to selective CD4 knockout mutants displayed on yeast.
Multi-mutant constructs MM3 (blue) and MM5 (red), stripped core (black), and stripped
core D368R (dashed black) are displayed on yeast, and binding to various antibodies is
assayed by flow cytometry: (a) gp120-specific mouse antiserum; (b) VRC01; (c) b12; (d)
b13. Mutations in multi-mutant constructs are listed in Table 3.

Table 1

Point mutations in defined mutant panel

Library point mutations			
N99Y	E269G	D397F	T455E
K121T	T278K	R403V	G459E
N197K	K282V	L405D	D461I
V200K	G327R	R412V	I467K
H216Y	Q337V	R419G	G473R
K231T	Q344V	G434K	R476V
T240K	S365K	Q442V	K490S
L265R	D368R	S446K	

Table 2

Mutations that disrupt binding to CD4 binding site-directed antibodies.

Antibody	Highly-disruptive mutations	Weak mutations
VRC01	D368R	S365K T455E G459E D461I G473R
b12	D368R R419G T455E	K282V S365K I467K
b13	D368R	R419G

Table 3

Point mutations in multi-mutant (MM) constructs for selective knockout of CD4 binding site-directed antibodies.

Construct	Mutations
MM1	S365K D461I G473R
MM2	S365K G459E G473R
MM3	S365K T455E G473R
MM4	T455E G473R
MM5	G459E G473R
MM6	D461I G473R