Human anti–HIV-neutralizing antibodies frequently target a conserved epitope essential for viral fitness

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The identification and characterization of conserved epitopes on the HIV-1 viral spike that are immunogenic in humans and targeted by neutralizing antibodies is an important step in vaccine design. Antibody cloning experiments revealed that 32% of all HIV-neutralizing antibodies expressed by the memory B cells in patients with high titers of broadly neutralizing antibodies recognize one or more "core" epitopes that were not defined. Here, we show that anti-core antibodies recognize a single conserved epitope on the gp120 subunit. Amino acids D474, M475, R476, which are essential for anti-core antibody binding, form an immunodominant triad at the outer domain/inner domain junction of gp120. The mutation of these residues to alanine impairs viral fusion and fitness. Thus, the core epitope, a frequent target of anti–HIV-neutralizing antibodies, including the broadly neutralizing antibody HJ16, is conserved and indispensible for viral infectivity. We conclude that the core epitope should be considered as a target for vaccine design.

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Abbreviations used: CD4bs, CD4 binding site; CD4is, CD4 induced site; EBV, Epstein-Barr virus; SPR, surface plasmon resonance; VL, variable loop.

A fraction of patients infected with HIV-1 develop broadly neutralizing antibodies against the virus (McMichael et al., 2010). In vitro studies indicate that these antibodies can reduce infectivity by interfering with virus–target–cell interactions or by blocking viral fusion (Dimmock, 1993; Robbins et al., 1995; Shibata et al., 1999; Zolla-Pazner, 2004). In addition, passive administration of mABs with broadly neutralizing activity to macaques or humans can provide sterilizing immunity or delay HIV-1 rebound (Emini et al., 1992; Gauduin et al., 1995; Mascola et al., 2000; Trkola et al., 2005). Therefore, it is generally believed that reproducing this type of serologic activity by immunization would be important for the development of an effective HIV vaccine (Stamatatos et al., 2009).

Although several different broadly neutralizing mABs that target HIV-1 envelope epitopes have been described (Zolla-Pazner, 2004; Burton et al., 2005), there have been few comprehensive efforts to clone and characterize the antibodies from patients with broadly neutralizing serologic responses. In an effort to understand the human antibody response to HIV-1, we cloned 502 anti-HIV-1 gp140 antibodies from the memory B cell compartment of six individuals with variable viral loads and high titers of broadly neutralizing antibodies (Scheid et al., 2009). We found that the memory B cell response to gp140 is composed of high affinity antibodies binding to the gp120 variable loops (VLs), the CD4 binding site (CD4bs), the induced coreceptor-binding site (CD4is), several different epitopes on gp41 (Pietzsch et al., 2010), and a group of potentially heterogeneous antibodies to one or more epitopes near the CD4bs, termed "core" (Scheid et al., 2009). The core antigen was not characterized molecularly; however, antibodies to this region accounted for 18% of all anti-gp140

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antibodies and 32% of all antibodies with neutralizing activity (Table S1; Scheid et al., 2009). Anti-core was the largest single group of neutralizers in the six patients studied. In addition, antibodies with characteristics similar to anti-core antibodies were also reported in a collection of mABs obtained from EBV-transformed B cells ("partial CD4 binding site" antibodies; Corti et al., 2010).

Anti-core antibodies bind to gp120, $gp120_{core}$ (a mutant that lacks V1-V3; Kwong et al., 1998), $gp120_{D368R}$ (which interferes with binding by CD4 and anti-CD4bs antibodies; Olshevsky et al., 1990; Thali et al., 1991; Pantophlet et al., 2003; Li et al., 2007), and $gp120_{I420R}$ (a mutant that interferes with the binding of anti-CD4–induced site [CD4is] antibodies; Thali et al., 1993). Anti-core antibodies do not bind to a stabilized $gp120_{core}$ protein that retains CD4 and b12 binding sites, but is mutated to reduce the flexibility of gp120 to improve presentation of conserved but discontinuous epitopes (Zhou et al., 2007; Scheid et al., 2009). Furthermore, anti-CD4bs and some anti-CD4is antibodies inhibit the binding of anti-core antibodies, suggesting that anti-core antibodies recognize an epitope that is closer to the CD4bs than to the CD4is (Scheid et al., 2009).

Here, we report on the characteristics of this new epitope. The data show that anti-core antibodies target a conformational epitope on gp120 found within the α5-helix of the molecule, which is highly conserved across different HIV-1 clades. This high degree of conservation correlates to viral fitness, as mutating the epitope results in loss of infectivity.

RESULTS

Fine mapping of anti-core antibodies cloned by single cell sorting

To map the epitope or epitopes recognized by anti-core antibodies, we assayed all anti-core antibodies for binding to 72 different alanine mutants of HIV-1 gp120 by ELISA. Controls included the anti-CD4bs antibody b12 (Burton et al., 1994; Saphire et al., 2001) and an anti–variable-loop antibody (1–79; Scheid et al., 2009). Mutations that reduced antibody binding to 60% or less compared with the WT protein were considered significant. The mutated residues were primarily spread across gp120 to cover a broad range of candidate binding sites, and then refined based on initial binding results. In particular, we included residues from the variable-loop 2 (VL2), the silent face, the CD4bs, the CD4is, the Phe 43-cavity (Kwong et al., 1998), in addition to residues that lie proximal or distal to these sites (Fig. 1 A and Fig. S1).

We found nine mutations that altered the binding of nearly all anti-core antibodies and b12, despite being physically distant from the CD4bs recognized by b12. Based on their position and physical chemical characteristics, these residues (L288, I449, T450, L265A, S264, C378, N262, F383, and F376) appear to be required to maintain the structural integrity of the molecule.

As previously demonstrated, alanine substitutions in the CD4bs (E370A, D368A, and D368A/E370A), or in close proximity to the CD4bs (N276A, R480A, and Y384A) reduce binding of the anti-CD4bs antibody b12 (Pantophlet et al., 2003) and CD4, respectively (Olshevsky et al., 1990; Fig. 1, A and B). These mutations had little or no effect on anti-core antibody binding (Fig. 1, A and B). Thus, the binding characteristics of the anti-CD4bs and anti-core antibodies are distinct. However, as expected from the antibody blocking experiments (Corti et al., 2010; Scheid et al., 2009), anti-core and anti-CD4bs antibody binding overlapped to a significant extent, with varying levels of sensitivity for F376A, P470A, W96A, E275A, and D477A (Fig. 1, A and B).

Among the 72 mutants, we found three mutations (D474A, M475A, and R476A) that inhibited the binding of anti-core antibodies, but had no significant effect on b12 (Fig. 1 C). These adjacent residues are in close proximity to the CD4bs and cover a stretch from a direct CD4 contact residue (D474) up to the α5 helix at the outer-domain/inner-domain junction of gp120 (M475, R476; Fig. 1 E). Among the 24 anti-core antibodies, only 2 were insensitive to these 3 mutations. Of the remaining 22, 16 were sensitive to both D474A and R476A (Fig. 1 D); in addition, 8 of these also showed altered binding to M475A (Fig. 1 D).

To determine whether anti-core antibodies recognize a linear peptide epitope containing D474, M475, and R476 we performed ELISAs with the DTNGTEIFRPGGG DMRDNWR peptide. Controls included a V3-loop peptide (NNNTRKSINIGPGRALYTT) that was recognized by an anti-V3-loop antibody (2–59; Scheid et al., 2009). We found that none of the 24 anti-core antibodies recognized this peptide (Fig. S2). Thus, anti-core antibodies recognize a conformational epitope containing amino acids D474, M475, and R476 in gp120 (Fig. 1, A–E).

Antibodies from EBV-transformed B cells

mABs with properties similar to anti-core that were able to completely or partially block the binding of sCD4 to Env-coated ELISA plates, henceforth defined as CD4bs- or partial CD4bs–specific mAbs were recently obtained from EBV immortalized memory B cells (Corti et al., 2010). To determine whether these antibodies (HGF12, HGI46, HGS2, HGW26, HGZ1, and HJ16) show binding characteristics similar to anti-core antibodies, we assayed all anti–partial CD4bs antibodies for binding to core epitope alanine mutants by ELISA (Fig. S3). Two of the antibodies (HGF12 and HGI46) could not be mapped; two others (HGW26 and HGZ1) were sensitive to D368A/E370A mutation and are likely to be conventional anti-CD4bs antibodies. HGS2 and HJ16 were sensitive to D474A, R476A, and M475A/R476A, but not to D368A; therefore, they target the same epitope as anti-core antibodies. Among these antibodies, HJ16 is of special interest because it is a broad and potent HIV neutralizer with very peculiar features, such a preferential neutralization of isolates not neutralized by b12 and an almost exclusive capacity to neutralize tier 2 isolates (Corti et al., 2010).

Trimeric BaL gp140

To confirm the mapping studies on trimeric gp140 of a different HIV strain, we produced BaL gp140 trimer and

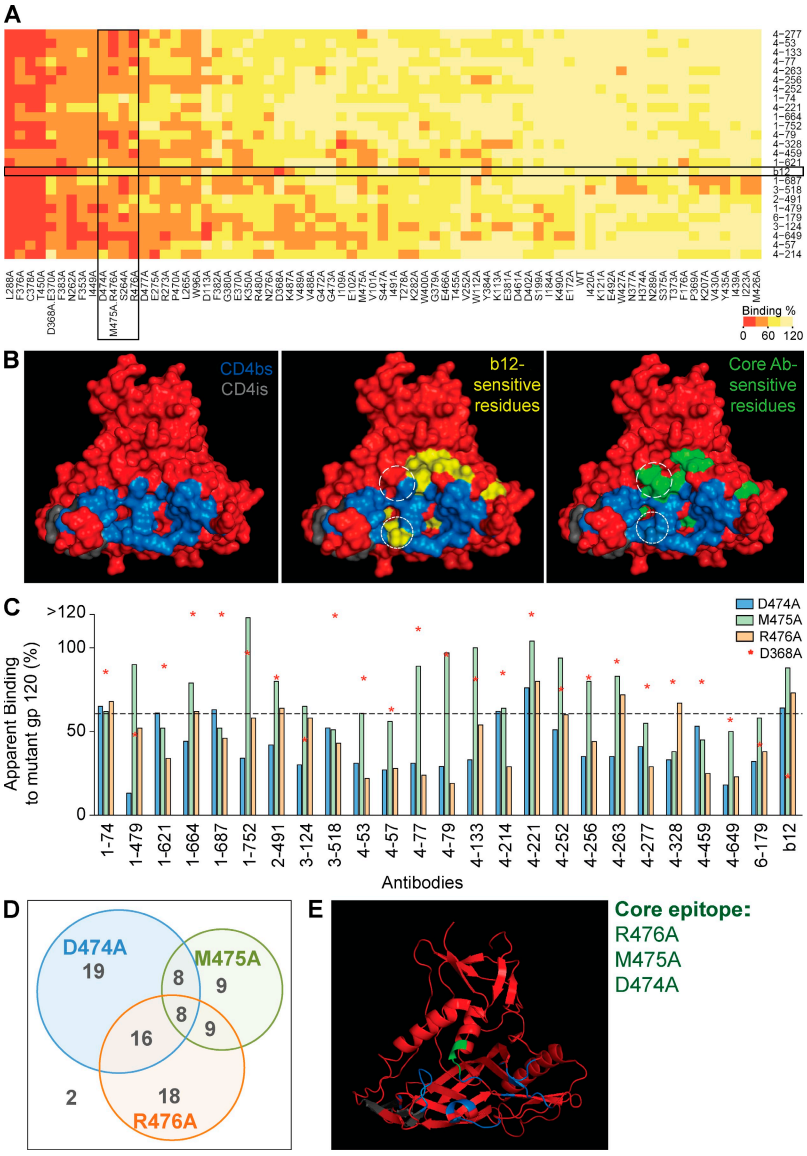

Figure 1. Mapping of the HIV-1 gp120 core epitope. (A) Heat map summarizes the binding of the different anti-core antibodies and b12 to gp120 alanine mutants. Red and orange fields indicate <60% binding, whereas yellow shows no difference compared with the WT gp120 control. The mapping of the anti-core epitope has been confirmed by three independent experiments. (B) Surface diagram of gp120 (PDB ID: 3DNO; Liu et al., 2008) showing the CD4bs (blue), the CD4is (gray), the b12 binding sites (yellow), and the core epitope (green). Residues that distinguished between the anti-CD4bs epitope and anti-core epitope are highlighted with circles. (C) Diagram shows the apparent binding of anti-core antibodies and b12 to mutant gp120$_{D474AM475AR476A}$ relative to gp120 WT in percent. The red star indicates binding to D368A. (D) Venn diagram summarizes the sensitivity for anti-core antibodies binding to D474A, M475A, and R476A. (E) Ribbon diagram of gp120 (PDB ID: 3DNO; Liu et al., 2008) shows the CD4bs (blue), the CD4is (gray), and the core epitope (green).

Binding to cell surface BaL gp160Δc trimer

The gp120 monomer and the artificial gp140 trimer lack yet to be defined features found on cell surface expressed gp160 viral spikes. To examine anti-core antibody binding to membrane-anchored molecules, we measured binding to a native gp160 trimer lacking the cytoplasmic domain of the spike (gp160Δc) expressed on the surface of BOSC.23 cells. Anti-core antibodies (4–79 and HGS2) bind the gp160Δc trimer, but not the mutant trimer (BaL gp160Δc$_{D474A/M475A/R476A}$). In contrast, b12 and the anti-VL antibodies bound to both gp160Δc and the gp160Δc mutant membrane anchored proteins. We conclude that the core epitope is accessible on the functional and fusion competent (see below) form of the HIV-1 spike and that it differs from the epitope recognized by the b12 anti-CD4bs antibody (Fig. 2 C).

corresponding core epitope mutant (BaL gp140$_{D474A/M475A/R476A}$) and repeated the ELISAs with two randomly selected anti-core antibodies (4–79 and HGS2), b12, and anti-VL controls. All of the antibodies tested showed similar binding characteristics on gp120 and the gp140 trimers (Fig. 2 A and Fig. S4).

To obtain a more precise measurement of the affinities of the antibodies to WT and mutant trimeric gp140 we performed surface plasmon resonance (SPR) experiments. The two anti-core antibodies, 4–79 and HGS2, bound to WT gp140 with high affinity K_As of 3.8×10^9 and 1.4×10^{10}, respectively, but binding to the mutant gp140$_{D474A/M475A/R476A}$ could not be detected. In contrast, control antibodies targeting the VL (2–1092) and the CD4bs (b12) retained their high-affinity binding to gp140$_{D474A/M475A/R476A}$, with K_As ranging from 9.6×10^8 to 4.8×10^9 (Fig. 2 B, Fig. S5, and Table S2).

Core epitope conservation

Given that most anti-core antibodies from different patients recognize a common epitope, we explored the possibility that D474, M475, and R476 might be conserved across different HIV-1 strains. Among 1,963 sequences analyzed from all clades (QuickAlign; http://www.hiv.lanl.gov), 56% contained all three of these residues and 35% of the remaining showed strong homology. Thus, the core epitope shows a moderate degree of precise conservation based on the primary amino acid sequence, but is highly conserved if homology is considered (Table S3).

Fusion and infectivity

To elucidate the possible roles of D474, M475, and R476 in fusion we developed an assay to measure fusion between BOSC.23 cells coexpressing BaL gp160Δc trimer and GFP,

with TZM.bl cells expressing red fluorescent protein (mCherry). Fusion between TZM.bl and BOSC.23 cells expressing the gp160Δc trimer, but not empty control GFP+ BOSC.23 cells, was readily detected by flow cytometry (Fig. 3 A). In contrast, there was little if any fusion of cells expressing the mutant spike (BaL gp160$\Delta c_{D474A/M475A/R476A}$).

To confirm that the events detected by flow cytometry represent fusion as opposed to binding, we repeated the experiments and analyzed them by image-based flow cytometry (ImageStream100; Amnis). Fusion was the predominant event when BOSC.23 cells expressed the gp160Δc trimer (Fig. 3 B). We conclude that D474, M475, and R476 are required for optimal fusion.

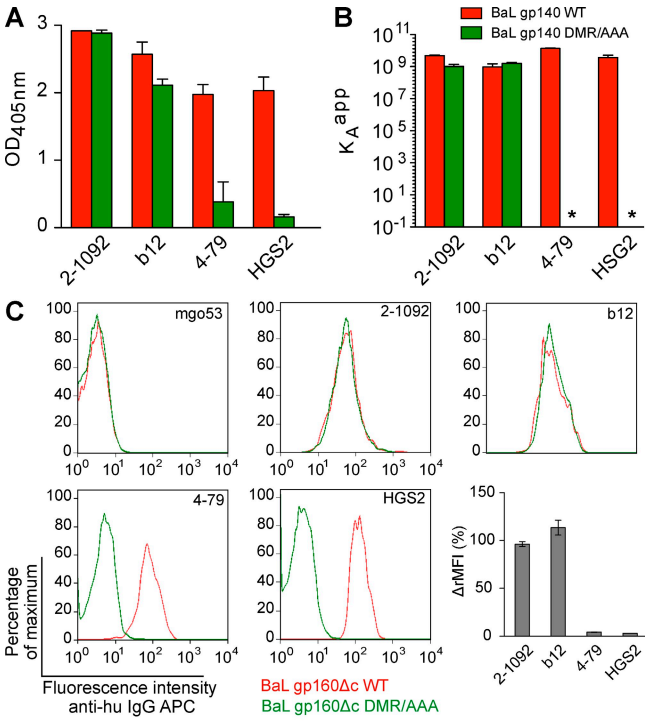

Figure 2. Binding to soluble and cell surface trimeric gp160Δc. (A) Graphs show optical density at 405 nm (OD_{405nm}) for the selected IgG antibodies as measured by capture ELISA with purified BaL gp140 WT and BaL $gp140_{D474A/M475A/R476A}$. See also Fig. S4. Error bars represent the SD from at least two independent experiments. (B) Graphs show apparent K_A (K_A^{app}, M^{-1}) for the selected IgG antibodies as measured by surface plasmon resonance (SPR) on chips derivatized with BaL gp140 WT and $BaL_{D474A/M475AR/476A}$. See also Fig. S5 and Table S2. Error bars represent the SEM from at least two independent experiments. * indicates that no binding to BaL $gp140_{D474A/M475A/R476A}$ was detected. (C) (left) Histogram plots show the binding of the selected antibodies to BaL gp160Δc and BaL $gp160\Delta c_{D474A/M475A/R476A}$ expressed on GFP-positive BOSC.23 cells. Controls include mgo53 (Wardemann et al., 2003), mAbs 2–1092 (anti-VL) and b12 (anti-CD4bs). BOSC.23 cells gated on GFP^{high} expression. The number of binding events as percentage of the maximum was plotted against APC fluorescence intensity. (right) Graphs show apparent differences in the relative median fluorescence intensity (ΔrMFI) for the selected IgG antibodies between BaL gp160Δc and BaL $gp160\Delta c_{D474A/M475A/R476A}$. Error bars represent the SD from at least two independent experiments.

To investigate the role of D474, M475, and R476 in viral infectivity, we assayed for the infection of TZM.bl cells with HIV-1 YU-2 and BaL pseudo-virus mutants (Fig. 3 C). YU-2 $gp160_{D474A/M475A/R476A}$ pseudo viruses showed a decrease in infectivity of four orders of magnitude at constant p24 levels (Table S4). The BaL pseudo virus was more sensitive to the mutation of the core epitope. R476A and M475A/R476A mutants decreased infectivity by two and three orders of magnitude, respectively, and we could not detect infection with the D474A mutant.

Collectively, the data from cell fusion and pseudo-virus infection experiments indicate that residues D474, M475, and R476 are important for optimal viral infectivity.

DISCUSSION

Although strong evidence suggests that any antibody that can bind to the HIV spike will neutralize the virus, only a small number of epitopes that induced broadly neutralizing antibodies upon HIV infection have been defined. These include the α1→2 mannose residues recognized by 2G12 (Trkola et al., 1996; Scanlan et al., 2002), the central region of the CD4bs bound by b12 (Barbas et al., 1992), and the V1/V2

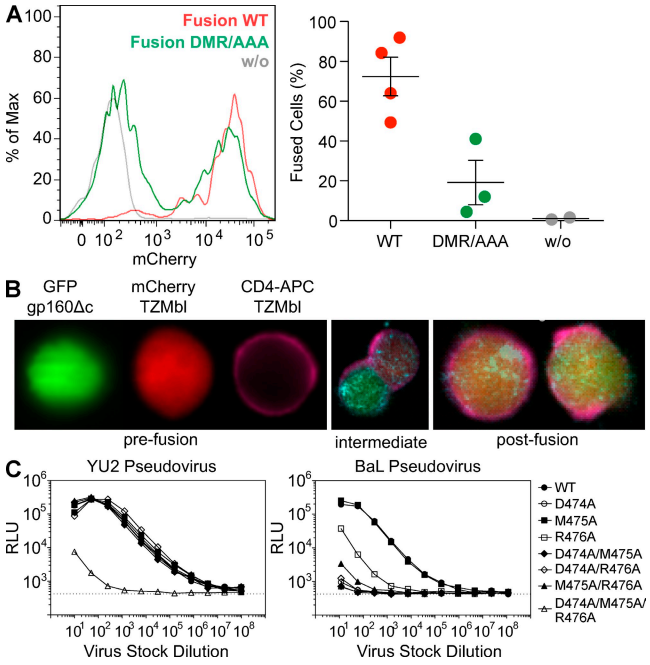

Figure 3. Fusion and infectivity. (A) (left) Histogram plots summarize fusion between BOSC.23 cells expressing GFP alone (w/o) or BaL gp160Δc or BaL $gp160\Delta c_{D474A/M475A/R476A}$ with mCherry expressing TZM.bl cells. (right) Fusion events (%) with mean and standard deviation for at least two independent experiments. (B) Image-based analysis of fusion. (left) Single staining compensation controls for BOSC.23 cells expressing BaL gp160Δc and GFP, mCherry expressing TZM.bl cells and CD4-APC stained TZM.bl cells. (middle) An intermediate fusion event. (right) The colocalization of the GFP-mCherry-APC signal after fusion. Experiment has been performed twice. (C) Graphs show the titration curves for TZM.bl cell infection by YU2 and BaL pseudoviruses, which has been performed twice. RLU relative light unit.

and V3 loops recognized by PG9 and PG16 (Walker et al., 2009; Walker and Burton, 2010). Unfortunately, vaccination approaches using recombinant HIV envelope proteins and derivatives specifically engineered to elicit broadly neutralizing antibodies have all been disappointing to date. Thus, the identification of additional immunogenic targets of neutralizing antibodies on the HIV viral spike remains an important issue in HIV vaccine research (Mascola and Montefiori, 2010). Optimal vaccine epitopes should be immunogenic in humans, conserved among HIV isolates and required for optimal viral infectivity. The core epitope fits all of these criteria.

Anti-core antibodies represent 18% of all anti-gp140 antibodies cloned from the memory B cell compartment of patients with high titers of broadly neutralizing antibodies (Scheid et al., 2009), but most importantly, they represent the most frequent neutralizers, accounting for 32% of all HIV-1 neutralizing antibodies (Scheid et al., 2009). In contrast, 18% of the neutralizing antibodies cloned in the same study showed binding properties similar to the anti-CD4bs antibody b12, 27% were anti-VL directed, and 23% recognized the CD4is (Scheid et al., 2009).

Neutralizing antibodies targeting a region other than the "central region of the CD4bs" were found in the sera of patients with broadly neutralizing serologic activity (Li et al., 2007). Anti-core plus anti-CD4bs containing serum and IgG fraction eluted from $gp120_{core}$ (which lacks VL 1–3, and therefore the CD4is) was more potent in viral neutralization than the anti–CD4bs-antibody–enriched fraction alone (Li et al., 2007). Thus, the serologic data are in agreement with the antibody-cloning experiments; both indicate that anti-core antibodies contribute significantly to the overall neutralizing activity in human serum.

Most antibodies that bind to HIV neutralizing epitopes are restricted in their neutralizing activity, including antibodies to the CD4bs. For example, among the antibodies that are like b12 and sensitive to the D368A mutation, most show neutralizing activity against tier 1 viruses, but much more limited activity against tier 2 viruses (Wyatt and Sodroski, 1998; Mascola, 2003; Pantophlet and Burton, 2006; Karlsson Hedestam et al., 2008; Scheid et al., 2009; Stamatatos et al., 2009). Similarly, most anti-core antibodies are not broad but are instead restricted to neutralize one or another strain of HIV-1. However, like the traditional anti-CD4bs antibodies, an occasional anti-core antibody, HJ16 exhibits a breadth of neutralizing activity comparable to, and generally complementary to b12, being able to neutralize approximately one third of all HIV-1 isolates tested, irrespective of clade (Corti et al., 2010). HJ16 also showed selective neutralization of tier 2, and not tier 1, isolates, making it particularly interesting as a template for vaccine design (Corti et al., 2010). Moreover, HJ16 was obtained from a clade C–infected patient, whereas the antibodies we cloned were obtained from clade B infected patients; therefore, the core epitope is immunogenic in humans even across different HIV isolates and clades.

Anti-core antibodies recognize a common epitope in close proximity to the CD4bs. We would therefore like to suggest that this group of antibodies should be referred to anti-CD4bs/DMR. Although this group of antibodies recognizes the same epitope, they are heterogeneous with respect to their neutralizing breadth and potency (Scheid et al., 2009; Corti et al., 2010). Similarly, traditional anti-CD4bs antibodies sensitive to the D368R mutation also show a wide range of neutralizing activity (Chen et al., 2009; Scheid et al., 2009; Corti et al., 2010). The structural basis for the difference is not entirely clear for either of the two classes of antibodies despite extensive structural information on the more traditional CD4bs antibodies. For example, there were only subtle differences between the structures of CD4bs antibodies F105 and b12 bound to a fragment of gp120, despite enormous differences in neutralizing activity (Chen et al., 2009). Further understanding of the molecular basis of differences in the neutralizing activity of these antibodies may require determining the structure of their cocrystals with native gp160.

Anti-CD4bs/DMR antibodies recognize a conformational epitope that includes the α5 helix at the outer-domain–inner-domain junction of gp120. The core epitope is highly conserved across different HIV-1 isolates and is essential for optimal infectivity, and therefore for viral fitness. Importantly, anti-CD4bs/DMR antibodies resemble b3 and b6 antibodies that were isolated as Fab fragments from a phage display library and lack neutralizing activity (Burton et al., 1991; Barbas et al., 1992; Pantophlet et al., 2003). Based on the comparative analysis of the structure of b12, which is broadly neutralizing, and b6, which is not, it has been suggested that a stabilized core protein, which does not allow b6 (Pantophlet et al., 2003; Zhou et al., 2007) or anti-core antibody (Scheid et al., 2009) binding, should be used as a vaccine immunogen (Dey et al., 2007, 2009; Mörner et al., 2009). However, antibodies to the core epitope can account for a significant fraction of the neutralizing activity in sera (Li et al., 2007), and of the antibodies produced by memory B cells (Scheid et al., 2009; Corti et al., 2010), including potent and broadly neutralizing antibodies (Corti et al., 2010). Therefore, we would like to suggest that an optimal HIV vaccine immunogen may require inclusion of the core epitope.

MATERIAL AND METHODS

mABs. Anti-human HIV-1 gp140 antibodies were described in (Barbas et al., 1992; Wardemann et al., 2003; Tiller et al., 2008; Scheid et al., 2009; Corti et al., 2010). All IgGs were expressed by cotransfection in HEK-293T cells (American Type Culture Collection). All work with human samples was performed in accordance with approved Institutional Review Board protocols (Scheid et al., 2009).

Mutagenesis and expression of recombinant gp120. Amino acid numbering refers to the HXB2CG reference sequence (Korber et al., 1998). Alanine mutations were introduced into pYU2gp120 (obtained from J. Sodroski, Dana-Farber Cancer Institute, Harvard School of Public Health, Boston, MA) using the QuickChange Lightning Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions. Mutagenesis primers were designed using Stratagene's web-based QuickChange primer design program. All site-directed mutations generated in this study were verified by DNA sequencing.

Recombinant proteins were produced in HEK-293T cells (American Type Culture Collection) grown in Dulbecco's modified Eagle's medium

(Invitrogen) supplemented with 1% (vol/vol) antibiotic-antimycotic (Invitrogen), 1% (vol/vol) L-glutamine (Invitrogen), and 1% (vol/vol) nutridoma-sp (Roche) that were transiently transfected with WT or mutant pYU2gp120 plasmids (50 µg). 3 d after transfection, culture supernatants were harvested and stored at 4°C and supplemented with complete protease inhibitor cocktail tablets (Roche) until use in ELISA.

ELISAs. To determine concentrations of the expressed gp120 proteins, ELISA plates (high-binding capacity; Corning) were coated with affinity-purified sheep anti–HIV-1gp120 antibody (Aalto Bio Reagents) recognizing a linear peptide at the carboxy-terminus (497–511; APTKAKRRVVQREKR) in PBS overnight at 5 µg/ml. The gp120 mutants were captured and detected with an anti-VL antibody (1–79; Scheid et al., 2009). The protein concentration of the gp120 mutants was determined using a gp120 standard and adjusted to 20 µg/ml.

To determine the relative binding of anti-core antibodies to the different gp120 mutants (MUT) compared with gp120WT, ELISA plates were coated as described in the previous paragraph. After washing with ultra pure water and blocking with 2 mM EDTA and 0.05% Tween-20 in PBS for 30 min at room temperature, mutant proteins were captured at 20 µg/ml for 2 h. Anti-core antibodies were incubated with the washed plates at serial dilutions in PBS-10% sheep serum (Equitech-Bio). Bound antibodies were detected with peroxidase conjugated affinity purified goat anti-Human IgG (Jackson ImmunoResearch Laboratories) using an HRP substrate kit (Bio-Rad Laboratories). The ELISA graphs were used to determine the linear range of antibody binding. The optical density (OD_{405nm}) for binding of a given antibody to gp120MUT divided by the OD_{405nm} of the same antibody to gp120WT was considered the percentage of apparent binding.

Peptide ELISA. Peptide ELISAs were performed as previously described (Mouquet et al., 2006). In brief, ELISA plates (Corning) were coated with 50 µl of a peptide containing the core epitope (underlined; DTNGTEIFRPGGGDMRDNWR) at 5 µg/ml, in PBS over night at room temperature. After washing three times with PBS-0.1% Tween 20 (PBST), wells were blocked with 1% PBS, 5% Tween20, and 3% sucrose milk powder for 1 h at room temperature. Serial dilutions of anti-core antibodies (starting at 4 µg/ml in PBST-1% BSA) were added, incubated for 1 h at room temperature, and visualized with peroxidase-conjugated affinity purified goat anti–human IgG (Jackson ImmunoResearch Laboratories) using an HRP substrate kit (Bio-Rad Laboratories). Controls included a V3-loop peptide (NNNTRKSINIGPGRALYTT) that was recognized by an anti-V3-loop antibody (2–59).

Mutagenesis and expression of recombinant gp140. Codon-optimized human BaL gp160 plasmid (pSF219; obtained from A.L. DeVico, University of Maryland, Baltimore, MD) was subcloned into pCDNA3.1. Mutations at the proteolytic cleavage site were introduced (arginines 508 and 511 to serines), as well as the trimerization domain from the C terminus of bacteriophage T4 fibritin inserted, thus leading to pBaLgp140Δ683(-/FT) (Yang et al., 2002). Alanine mutations (D474A/M475A/R476A) were introduced as described and confirmed by sequencing. Recombinant proteins were produced in HEK-293T cells as described and purified by lectin and nickel-chelate affinity chromatography (Freeze, 2001; Petty, 1996).

Surface plasmon resonance. All experiments were performed with a Biacore T100 (Biacore, Inc.) in HBS-EP+ running buffer (Biacore, Inc.) at 25°C. Samples were analyzed in kinetic experiments performed at least in duplicate. BaL gp140 WT and DMR/AAA mutant proteins at 62.5 µg/ml were immobilized on CM5 chips (Biacore, Inc.) by amine coupling at pH 4.5, resulting in an immobilization level of 5,000 RUs. For kinetic measurements on the gp140 WT- and gp140 DMR/AAA-derivatized chips, IgGs were injected through flow cells at 333 nM and 4 successive 1:2 dilutions in HBS-EP+ running buffer (Biacore, Inc.) at flow rates of 40 µl/min with 3-min association and 5-min dissociation. The sensor surface was regenerated between each experiment with a 30-s injection of 10 mM glycine-HCl, pH 2.5, at a flow rate of 50 µl/min. Off rate (s^{-1}), on rate ($k_{a1}[M^{-1}s^{-1}$, $k_{a1}[RU^{-1}s^{-1}]$), and binding constants ($K_{A1}[M]$, $K_{A2}[RU^{-1}]$) were calculated after subtraction of backgrounds (binding to a control flow cell and signal of the HBS-EP+ running buffer) using Biacore T100 Evaluation software using the kinetic analysis and the bivalent model. K_{A1} value was used as an estimation of apparent K_A (K_A^{app}) because K_{A1} represents the majority of the binding.

Expression of the native gp160Δc trimer and FACS-based binding assay. Codon-optimized human BaL gp160 plasmid (pSF219; obtained from A.L. DeVico, University of Maryland, Baltimore, MD), whose intracellular tail was deleted (gp160Δc), was subcloned into pMX-IRES-GFP (Robbiani et al., 2008). Alanine mutations (D474A/M475A/R476A) were introduced as described in the previous paragraph and confirmed by sequencing. Human embryonic kidney BOSC.23 cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% (vol/vol) fetal calf serum, 1% (vol/vol) antibiotic-antimycotic (Invitrogen), and 1% (vol/vol) L-glutamine (Invitrogen). FuGENE 6 (Roche) was used for transient transfection according to the manufacturer's instructions. 24 h later, cells were harvested with enzyme-free cell dissociation buffer (Invitrogen) and stained with dialyzed human mABs (2–1092, 4–79, HGS2, mgo53, and b12) at 0.4 µg/ml for 30 min at 4°C. Primary antibodies were visualized with APC-conjugated mouse anti–human IgG antibody (BD) according to the manufacturer's instructions. Flow cytometry was perfomed on FACSCalibur (BD). The median fluorescence intensity (MFI) was calculated using FlowJo software. The differences in relative MFI (ΔrMFI) between gp160Δc and MUT BaL gp160Δc were calculated according the following formula: $rMFI = 100 \times (1 - [\{MFI - MFI_{MUT}\}/MFI])$.

Fusion assay. BaL gp160Δc and gp160Δc D474A/M475A/R476A were expressed on BOSC.23 cells, harvested as described, and mixed with mCherry positive TZM.bl cells in excess. The cell suspension was centrifuged at 657× g and 37°C for 1 h and incubated for one additional hour at 37°C and 5% CO_2, then treated with 0.05% Trypsin-EDTA. Cells were assayed on ImageStream100 (Amnis) and BD LSR II (BD Biosciences) and analyzed with FlowJo software. Fused cells (%) = $n(GFP^+mCherry^+)/ [n(GFP^+mCherry^+) + n(GFP^+mCherry^-)]$.

HIV-1 pseudovirus infectivity assay. Alanine mutations were introduced in pYU-2Env and pBaL-Env as described above. Clones were cotransfected with pHIVΔEnv in HEK-293 T cells. The virus stocks were titrated on TZM.bl cells to measure the median tissue culture infective dose ($TCID_{50}$; Li et al., 2005). p24 ELISAs (ZeptoMetrix) were performed according to manufacturer's instructions.

Online supplemental material. The supplemental material includes results of the mapping ELISA (Figs. S1, 3, and 4), the peptide ELISA (Fig. S2), SPR sensograms (Fig. S5), TZM.bl neutralization assay (Table S1), SPR affinities (Table S2), conservation of the core epitope (Table S3), and the pseudo virus infectivity assay (Table S4). Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20101176/DC1.

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