Mechanism of HIV-1 Neutralization by Antibodies Targeting a Membrane-Proximal Region of gp41

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Running title: Gp41-specific neutralizing antibodies

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Abstract

Induction of broadly neutralizing antibodies (bNAbs) is an important goal for HIV-1 vaccine development. Two autoreactive bNAbs, 2F5 and 4E10, recognize a conserved region on the HIV-1 envelope glycoprotein gp41 adjacent to the viral membrane known as the “membrane-proximal external region” (MPER). They block viral infection by targeting a fusion-intermediate conformation of gp41, assisted by an additional interaction with viral membrane. Another MPER-specific antibody, 10E8, has recently been reported to neutralize HIV-1 with potency and breadth much greater than those of 2F5 or 4E10, but it appeared not to bind phospholipids, and might target the untriggered envelope spikes, raising the hope that the MPER could be harnessed for vaccine design without major immunological concerns. Here we show that 10E8 indeed binds lipid bilayers through two hydrophobic residues in its CDR H3 by three independent approaches. Its weak affinity for membranes in general and preference for cholesterol-rich membranes may account for its great neutralization potency, as it is less likely than other MPER-specific antibodies to bind cellular membranes non-specifically. 10E8 binds with high-affinity to a construct mimicking the fusion intermediate of gp41, but fails to recognize the envelope trimers representing the untriggered conformation. Moreover, we can improve potency of 4E10 without affecting its binding to gp41 by a modification of its lipid-interacting CDR H3. These results reveal a general mechanism of HIV-1 neutralization by MPER-specific antibodies that involves interactions with viral lipids.
Introduction

Fusion of viral and target cell membranes mediated by viral envelope glycoprotein allows HIV-1 to enter host cells (16). The mature envelope spikes (trimeric (gp160), cleaved to (gp120/gp41)) are the sole viral antigens on the virion surface. Conformational changes in gp120 when triggered by binding to receptor (CD4) and co-receptor (e.g., CCR5 or CXCR4) lead to a cascade of refolding events in gp41, in which its N-terminal fusion peptide translocates and inserts into the host cell membrane. A proposed transient, extended conformation of gp41, with the fusion peptide in the host cell membrane and the transmembrane anchor still in the viral membrane, has been called the “prehairpin intermediate” (6). It is the target of HIV-1 entry inhibitors, such as T-20/Enfuvirtide – the first approved fusion-inhibiting antiviral drug (20). Further transition from the fusion intermediate to the postfusion state of gp41 is believed to bring together the two membranes and ultimately to induce membrane fusion.

Strong antibody responses to the envelope glycoprotein are often detected in HIV-1 infected patients even during acute infection, but antibodies that can effectively block HIV-1 transmission or disease progression are rare, as most detected antibodies are non-neutralizing or strain-specific (7, 32, 38). Recent studies indicate that 10-25% of patients indeed produce broadly neutralizing antibodies (bNAb) at low levels during the course of HIV-1 infection (28, 35), raising the hope that search for immunogens that can induce stronger responses of this kind may lead to an effective vaccine. Major challenges to generating bNAb by vaccination include unprecedented sequence diversity, conformational flexibility, and a protective “glycan shield” of the envelope glycoprotein, as well as potential host immunoregulatory constraints and lack of reactivity by envelope immunogens to germline antibodies of known bNabs (17, 18, 23, 28, 32, 38). Thus, detailed studies of isolated broadly reactive neutralizing antibodies that recognize conserved regions of the envelope glycoprotein are critical for guiding strategies to mimic their corresponding immunogens.

Human bNAb 2F5 and 4E10 interact with the “membrane-proximal external region” (MPER) of gp41, the segment of the protein adjacent to the viral membrane (30, 36). Both block viral infection by attacking the prehairpin intermediate conformation of gp41.
with help of their ability to bind viral membrane (2, 13), although additional mechanisms of HIV-1 neutralization by these antibodies have also been suggested (21, 33). A useful MPER-based immunogen would therefore likely include a protein component to present the relevant gp41 conformation and a lipid component for inducing membrane binding. Autoreactivity of both 2F5 and 4E10 is, however, a potential barrier to effective induction of similar antibodies by a vaccine immunogen (17). Another MPER-specific neutralizing antibody, 10E8, has been isolated recently (19); it was reported not to bind either lipid bilayers or any other human autoantigens. It also showed better recognition of the envelope glycoprotein expressed on a cell surface than do 2F5 and 4E10, igniting excitement that the MPER might be harnessed to induce protective antibody responses without major concerns regarding the unwanted immunological limitations associated with 2F5 and 4E10 (17). The reported accessibility of the 10E8 epitope on the native envelope spikes is much lower, however, than the epitopes for antibodies targeting the untriggered envelope with similar neutralization potencies (19). Moreover, the affinity of 10E8 variants for gp41 does not fully correlate with their neutralizing strength (19), suggesting that a better understanding of the action of this antibody is needed to guide vaccine-design efforts for inducing 10E8-like antibodies.

In this study, we show that 10E8 does bind lipid bilayers through two hydrophobic residues in its CDR-H3 (third heavy-chain complementarity determining region). Its affinity for membranes is weak in general, but it has a preference for cholesterol-rich membranes that may account for its great potency in neutralization, as it is less likely than other MPER-specific antibodies to bind cellular membranes non-specifically. Moreover, it binds with high-affinity to gp41-inter, a construct designed to mimic the fusion intermediate of gp41 (13), but fails to recognize the epitope on envelope trimers representing the native, untriggered conformation. Furthermore, we can improve potency of 4E10 by a modification of its lipid-interacting CDR H3 without affecting its binding to gp41. Thus, MPER bNAbs are likely to neutralize by a common mechanism. These results have important implications for the MPER-based vaccine design.
Materials and Methods

Production of antibodies and recombinant proteins

The expression constructs of 10E8 and a small amount of purified 10E8 antibody were obtained from the NIH AIDS reagent program (heavy chain (Cat# 12290) and light chain (Cat# 12291); purified IgG (Cat# 12294)). The 10E8 mutants (F100aA, W100bA, F100aA-W100bA and P100fA) were generated by PCR-based mutagenesis and mutations were confirmed by DNA sequencing. The antibodies were expressed in 293T cells by transient transfection and purified by affinity chromatography using GammaBind Plus resin (GE Healthcare, Little Chalfont, UK) following protocols described previously(22). 10E8 concentration was measured by absorbance at 280 nm assuming that 1 OD = 1 mg/ml. The wildtype 10E8 and mutant P100fA have a low solubility at 4°C and were stored at a low concentration (~0.7 mg/ml). All antibodies were buffer-exchanged into PBS and filtered through Ultrafree centrifugal filters (0.22 µM; EMD Millipore, Billerica, MA) before use. The VRC01 expression construct was kindly provided by John Mascola (VRC, NIH); 17b hybridoma by James Robinson (Tulane University); 1281 hybridoma by Susan Zolla-Pazner (New York University). We have generated expression constructs of 2G12 IgG and 4E10 Fab using synthetic genes made by GeneArt (Life Technologies Corporation, Grand Island, NY). 4E10 Fab mutants were created by GENEWIZ, Inc. (South Plainfield, NJ). 4E10 IgG was purchased from Polymun (Klosterneuburg, Austria). Other intact antibodies or Fab fragments were produced in 293T cells or hybridoma following protocols as described (11). Production of soluble CD4, 92UG037.8 gp140 trimer and gp41-inter was described previously (12, 13, 22).

The 92UG037.8 gp41-post construct containing the following sequence:

AVVELGAVFIGFGLTAGSTMGAASITLTQVRKLSSGIVQGQSNLRAIEAQQHL

LKLTVGKQLQARVLAVERYLRDQQLGIGWGCSGKLICTTNVPWNSSWNKSE

REIWENMTWLDKEISNYTHIYELIEESQKQQEKNEQELLEDWKANLWNWF

DISNWLWYIKIFIMVGGLIGLRIVFAVLSV1 with an N-terminal Honeybee melittin signal sequence and a C-terminal his-tag was generated by PCR and cloned into the
expression vector pFastBac1 (Life Technologies Corporation). The gp41-post protein was expressed in insect cells using the Bac-to-Bac system (Life Technologies Corporation) as described(13). Briefly, recombinant baculovirus was generated according to the manufacturer’s protocol and amplified in Sf9 insect cells. For protein production, T. ni (Hi-5) cells (2x10^6 cells/ml) were infected at the optimal MOI and harvested 72 hours post-infection by centrifugation. The cell pellets were resuspended in PBS containing 0.5 mM PMSF and protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany), then subjected to three cycles of freeze-thaw and brief sonication to disrupt membranes. Soluble proteins were removed by centrifugation and membrane-containing pellets were extracted with a buffer containing 20 mM Tris-HCl, pH 8.0, 350 mM NaCl, 10% glycerol and 1% Fos-Choline-12 at 4°C for 24 hours. The extract was clarified by high-speed centrifugation (2x10^5 xg) and filtration through a 0.22 µm filter, diluted 4 fold with a solution containing 20 mM Tris-HCl, pH 8.0 and 150 mM NaCl, and then loaded onto a 5 ml HisTrap column (GE Healthcare) that was equilibrated in 20 mM Tris-HCl, 150 mM NaCl and 0.2% Fos-Choline-12. The histagged gp41-post protein was eluted from the HisTrap column with a buffer containing 300 mM imidazole, concentrated and further purified by gel filtration chromatography on a Superdex 200 column in a running buffer containing 20 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.2% Fos-Choline-12. Fractions containing the gp41-post trimers were pooled and incubated with 1281 Fab at room temperature for an hour. To replace Fos-Choline-12 with dodecyl maltoside and remove unbound 1281 Fab, the gp41-post-1281 complex was further purified on a Superdex 200 column in a buffer containing 20 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.1% dodecyl maltoside.

**Liposome preparation.** All lipids including chicken egg phosphatidylcholine (PC), chicken egg phosphatidylethanolamine (PE), porcine brain PS (phosphatidylserine), porcine brain sphingomyelin (SM), and bovine heart cardiolipin (CL) and ovine wool cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). Liposomes were prepared following a modified sonication-extrusion protocol as described (http://www.avantilipids.com/images/PDF/MorrisseyLabProtocolForPrepSuvBySonication.pdf). Briefly, lipids were dissolved in chloroform and mixed in a glass vial with various molar ratios, including PC:CL = 3:1 for PC-CL liposomes; and PC:PS = 3:1 for PC-PS
Liposomes; PC: PE: PS: SM: cholesterol = 1.1:2.3:1.0:2.2:5.5 for low-cholesterol HIV-1-like membrane; and PC: PE: SM: cholesterol = 1.2:2.6:1.0:2.4:11.6 for high-cholesterol HIV-1-like membrane. The lipid ratio for the high-cholesterol HIV-1-like membrane was chosen empirically based on liposome stability and signals for 10E8 binding. Chloroform was evaporated under a stream of N2 for ~10 min and lipids were further vacuum-dried overnight. Dry lipid films were then hydrated in PBS either on ice or at room temperature, followed by brief sonication for 20 seconds, and subjected to 21-25 cycles of extrusion through 0.2 µm polycarbonate filter membranes (GE Healthcare) using an Avanti extruder (Avanti Polar Lipids). Liposomes were usually used immediately, but stable for at least 5 days, as judged by dynamic light scattering (see below). For liposomes used for SPR experiments, 0.5% (molar ratio) biotin-labeled PE was included to allow capturing by streptavidin-coated (SA) sensor chips. In addition, the biotinylated liposomes were extruded through 0.05 µm polycarbonate filter membranes (GE Healthcare) for analysis by a Biacore instrument. To rule out the possibility that the observed binding of liposomes by antibodies was due to minor contaminants in purified natural phospholipids, we have produced liposomes by the same protocol using synthetic phospholipids, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), N-palmitoyl-D-erythro-sphingosylphosphorylcholine (SM), purchased from Avanti Polar Lipids, and obtained the same results.

Liposome floatation assay. 0.5 mg/ml of each antibody and 1 mg/ml liposomes prepared with 0.2 µm filters were mixed and incubated either at 37°C or on ice for an hour. In a centrifuge tube, 0.15 ml of the protein-liposome mixture was gently mixed with 0.3 ml of 70% (w/v) sucrose in PBS to give 0.45 ml of 46.7% (w/v) sucrose solution that formed the bottom layer. 1 ml of 25% (w/v) sucrose in PBS was carefully overlaid on the top of the 46.7% (w/v) sucrose solution as the middle layer, and 0.15 ml of 5% (w/v) sucrose in PBS was then overlaid as the top layer. After centrifugation at 52,000 rpm in a Beckman Optima TL Ultracentrifuge using a TLS55 rotor for 3 hours at either 37°C or 4°C, 6 fractions in 0.2 ml and one last fraction in 0.3 ml were collected from the top to the bottom of the sucrose gradient, and analyzed by western blot using an anti-human Fab antibody conjugated with horseradish peroxidase (Sigma-Aldrich, St. Louis, MO).
Dynamic light scattering (DLS) analysis. 0.5 mg/ml of liposomes prepared with 0.2 µm filters and 0.5 mg/ml of each antibody were preincubated at either 37°C or 20°C, and then mixed in a cuvette from which light scattering was immediately recorded in a DynoProtein Solution instrument (Wyatt Technology, Santa Barbara, CA). 50 consecutive acquisitions were averaged for each measurement. Program DYNAMICS V5 was used for data analysis.

Surface Plasmon Resonance (SPR) Binding Assays. All experiments were performed in duplicate or repeated at least twice with a Biacore 3000 (GE Healthcare). Interactions of antibodies with envelope constructs were analyzed using a single cycle kinetic (SCK) method, following a protocol as described (https://www.biacore.com/lifesciences/products/systems_overview/Biacore_T200/Training+Course/index.html), with a 1 min association phase and a 5 min dissociation phase without regeneration. Experiments to compare gp140 trimer, gp41-inter and gp41-post were carried out in a HBS buffer (10 mM HEPES, pH 7.0, 150 mM NaCl, 3 mM EDTA) containing 0.1% dodecyl maltoside. Each antibody IgG was captured on a sensor chip coated with Protein A to an immobilization level of 1,800 RU and envelope proteins at various concentrations (2.5-40 nM) were passed over at a flow rate of 30 µl/min without regeneration. All other protein-binding experiments were performed in a standard HBS-EP running buffer (10 mM HEPES, pH 7.0, 150 mM NaCl, 3 mM EDTA, 0.005% P20). Each antibody IgG was captured on a sensor chip coated with Protein A to an immobilization level of 2,000 RU and envelope proteins at various concentrations (2.5-40 nM for binding to 10E8, 2G12 and VRC01; 10-160 nM for binding to 10E8 mutants) were passed over at a flow rate of 30 µl/min without regeneration. Binding kinetics was evaluated using BiaEvaluation software (GE Healthcare) and all sensorgrams were fit to a 1:1 binding model. A script for analyzing SCK data obtained with Biacore 3000 was kindly provided by GE Healthcare.

Lipid binding experiments were carried out in PBS without detergent. PC-CL and HIV-1-like liposomes incorporated with 0.5% (molar ratio) biotin-PE were prepared using 0.05 µm filter membranes as described above. All liposome preparations were monitored for homogeneity by DLS analysis and were stable for at least 5 days. Biotinylated liposomes
were captured on a streptavidin (SA) chip (GE Healthcare) to an immobilization level of ~6,000 RU for HIV-1-like liposomes or ~3,000 RU for PC-CL liposomes. Each antibody IgG at a concentration of 1.0 µM was passed over the lipid surface at a flow rate of 50 µl/min. Sensorgrams were recorded with a 2 min association phase and a 5 min dissociation phase.

**Flow cytometry.** Transfection of 293T cells with the HIV-1 92UG037.8 gp160 was carried out as described (12). 40 hours posttransfection, transfected and control cells were detached from plates using PBS containing 1% BSA, and washed with ice-cold PBS containing 1% BSA. 10^6 cells/ml were incubated for an hour at 4°C with various anti-HIV-1 envelope monoclonal antibodies at 50 µg/ml in PBS containing 1% BSA, washed by PBS with BSA three time and then stained with 5 µg/ml of R-Phycoerythrin AffiniPure F(ab')2 fragment goat anti-human IgG, F(ab')2 Fragment specific secondary antibody (Jackson ImmunoResearch laboratories, West Grove, PA) in PBS containing 1% BSA. Labeled cells were then washed with cold PBS with 1% BSA three times and analyzed immediately using a BD FACScan instrument and program CellQuest (BD Biosciences, San Jose, California).

**Antibody neutralization assay in TZM.bl cells.** Antibody neutralization was measured using a protocol of the luciferase-based HIV-1 neutralization assay in TZM.bl cells as described (24, 27). Briefly, 5-fold serial dilutions of 10E8 or 4E10 were performed in duplicate (96-well flat bottom plate) in 10% DMEM growth medium (100 µl/well). TZM.bl cells were added (1x10^4/well in 50 µl volume) and the plates were incubated for 1 hour at 37°C. 10 selected HIV-1 Env pseudoviruses were then added (100 µl/well) in 10% DMEM growth medium containing DEAE-Dextran (Sigma-Aldrich) at a final concentration of 11 µg/ml. Assay controls included replicate wells of TZM.bl cells alone (cell control) and TZM.bl cells with virus (virus control). Following a 48 hour incubation at 37°C, 150 µl of assay medium was removed from each well and 100 µl of Bright-Glo luciferase reagent (Promega, Madison, WI) were added and luminescence measured using a Victor 3 luminometer (Perkin Elmer, Waltham, MA). The 50% inhibitory concentration (IC50) and 80% inhibitory concentration (IC80) were calculated based on
the antibody dilution that caused a 50% or 80% reduction in relative luminescence units (RLU) compared to the virus control wells after subtraction of cell control RLUs.

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Results

Production of 10E8 and its mutants

We set out to determine the conformational state of the envelope glycoprotein recognized by 10E8, and to understand why 10E8 is much more potent than 4E10 despite its lower affinity for a similar epitope on gp41 (19). We obtained the 10E8 expression constructs from the NIH AIDS reagent program, contributed by Huang and colleagues, expressed the 10E8 antibody in 293T cells, and purified it by protein G affinity chromatography. IC₅₀ and IC₈₀ neutralization titers of our preparation using ten selected HIV-1 isolates with a wide range of sensitivity to the antibody are largely concordant with values reported previously (Table S1), confirming that our antibody is indistinguishable from the reported preparation (19). The freshly purified 10E8 had an unusually low solubility (<1 mg/ml) for an immunoglobulin G (IgG) in phosphate buffered saline (PBS) and precipitated over time at 4°C, suggesting that there might be exposed hydrophobic surfaces causing aggregation. Close examination of the crystal structure of 10E8 in complex with the epitope peptide suggested that two hydrophobic residues, F100a and W100b, in the CDR H3 may be exposed in the unliganded form (19) and could interact with phospholipids (Fig. S1). We therefore produced three 10E8 mutants: F100aA (Phe to Ala), W100bA (Trp to Ala) and F100aA-W100bA (double mutant), to test the function of these residues. An additional mutant, P100fA, in the gp41-binding site was also generated as a control. The mutant antibodies were produced by the same protocol, but the first three mutants showed a much higher solubility (up to 10 mg/ml) in PBS after purification at 4°C, confirming our hypothesis that the low solubility of 10E8 correlates with its surface hydrophobicity.

Interactions of 10E8 with lipid bilayers
We used three methods to test whether 10E8 interacts with membrane lipids. The first was a liposome flotation assay with liposomes of three different compositions: phosphatidylcholine (PC) and cardiolipin (CL) (PC:CL = 3:1); PC and phosphatidylserine (PS) (PC:PS = 3:1); as well as a mixture mimicking the lipid composition of HIV-1 viral membrane (PC:phosphatidylethanolamine (PE):PS:sphingomyelin:cholesterol = 1.2:2.6:1.0:2.4:11.6; ref (4)). The high cholesterol level in the HIV-1-like liposomes enhanced signals in lipid-binding assays, although the liposomes with the composition used in published studies (PC:PE:PS:sphingomyelin:cholesterol = 1.1:2.3:1.0:2.2:5.5; ref (4)) also gave positive results (see below). Sensitivity of lipid composition may be significant, because HIV-1 membranes have been reported to have a cholesterol level 2-3 fold higher than that of plasma membranes (3, 4, 25) and their high cholesterol content is critical for viral infectivity and budding (15, 31). The experiments were carried out at both 37°C (Figs. 1 and S2) and 4°C (Fig. S3). As expected, the known membrane-binding antibody, 4E10, associated with liposomes of all three types after centrifugation in a sucrose gradient, while a gp120-specific antibody 17b that does not interact with membranes sedimented to the bottom of the gradient as free protein (Figs. 1, S2 and S3). Like 4E10, wildtype 10E8 also floated with both HIV-1-like and PC:CL liposomes, but not with PC:PS liposomes (Figs. 1, S2 and S3), indicating that this antibody binds phospholipids, with certain compositional preferences. Mutation of either of the two hydrophobic residues in the CDR H3 (F100aA or W100bA) led to much weaker binding to PC:CL liposomes and no detectable binding to HIV-1-like liposomes, while the double mutation (F100aA-W100bA) completely abolished binding to both types of lipid bilayers. The P100fA mutant still bound lipid bilayers as expected (Fig. S2). We conclude that the two hydrophobic residues, F100a and W100b, in the CDR H3 of 10E8 mediate its interaction with membrane.

As a second assay for membrane binding, we used dynamic light scattering to monitor crosslinking of liposomes in solution by intact, bivalent IgGs at both 37°C (Figs. 2 and S4) and 20°C (Fig. S5). Our freshly prepared liposomes had an average hydrodynamic radius of 100 nm, stable for at least 5 days and unperturbed by addition of PBS (Figs. 2,
S4 and S5). 10E8 and 10E8-P100fA, which tended to aggregate at high concentrations and low temperature, did not form large aggregates under the experimental conditions we used (Figs. S4A and S5A). As expected, addition of 17b IgG did not have any effect on the size of the liposomes under all conditions tested. In contrast, addition of lipid-binding 4E10 IgG led to a dramatic increase in the apparent size of the liposomes (up to 1x10^6 nm), for all three types of liposomes at both temperatures (Figs. 2, S4 and S5), consistent with the previous observations of strong membrane interaction by 4E10 (2, 17, 34).

Addition of 10E8 IgG produced a significant, but moderate, increase in the sizes of HIV-1-like liposomes at both 37°C and 20°C (Figs. 2 and S5B), and of PC-CL liposomes at 20°C (Fig. S5C), but no changes in the size of PC-PS liposomes at either temperature (Figs. S4C and S5D). A 10E8 preparation obtained from the NIH AIDS reagent program also gave the same results (Figs. S4B, S4C, S5B and S5C). Liposomes lack lipid asymmetry in biological membranes. Removing PE in the HIV-1-like liposomes with a lower cholesterol level to mimic the lipid composition of the outer leaflet of biological membranes significantly enhanced their binding to 10E8, while their strong binding to 4E10 was not sensitive to the change in composition (Fig. S6). These observations agreed with the notion that 10E8 interacts weakly with lipid bilayers, with a preference for the cholesterol-rich, HIV-1-like membrane. Mutation of the hydrophobic residues at positions 100a, and 100b in the 10E8 IgG (F100aA IgG, W100bA IgG and F100aA-W100bA IgG) did not affect the size of any liposomes under any of the conditions tested, nor did the monovalent 10E8 Fab. The mutant P100fA behaved similarly to wildtype 10E8 in this assay (Figs. S4 and S5). These results indicate that the large particles observed by dynamic light scattering are indeed the result of crosslinking of liposomes, not of antibody-mediated lipidosome fusion, and further confirm that the two hydrophobic residues in the CDR H3 mediate 10E8 interaction with lipid bilayers. These findings provide direct evidence that both 10E8 and 4E10 interact with lipid bilayers in solution.

As a third assay for lipid interaction, we used SPR (surface plasmon resonance) at both 37°C and room temperature (Figs. 3 and S7). Biotin-labeled PE was incorporated into liposomes that were then immobilized on the surface of a sensor chip by streptavidin, and various antibodies were passed over the liposome surfaces. As controls, 4E10 showed
high levels of binding to HIV-1-like and PC:CL liposomes; and gp120-specific antibodies, 17b and VRC01, did not bind detectably to any liposomes. 10E8 bound both types of liposomes, with a fast off-rate for the HIV-1-like membrane and a slow off-rate for the cardiolipin-containing membrane. Mutating the two hydrophobic residues reduced binding for both types of liposomes to an almost undetectable level. We observed a similar effect of these mutations, but with a weaker signal for HIV-1-like liposomes with a lower cholesterol level (Fig. S7). These results are consistent with the data obtained from the liposome flotation and DLS analyses.

10E8 neutralizes by targeting the fusion intermediate of gp41

To determine which conformational state of the envelope spikes 10E8 recognizes, we first analyzed direct binding of 10E8 to the envelope protein expressed on the surface of 293T cells using a FACS (fluorescence-activated cell sorting) assay. We chose to use a 10E8-sensitive, primary HIV-1 A-clade isolate, 92UG037.8, from which we have produced stable, monomeric preparations of the envelope glycoprotein to represent its distinct conformational states (12, 13, 22). We verified that the expressed protein was active by showing that it mediated cell-cell fusion. As shown in Fig. 4, one of the most potent CD4 binding site bNAbs, VRC01, which targets the untriggered, native conformation of the envelope trimer with a potency similar to that of 10E8, bound strongly to the cell-surface expressed 92UG037.8 envelope spikes, but not to the surface of control cells. In contrast, 4E10, which targets the prehairpin intermediate state of gp41, bound weakly, but detectably, to the cell surfaces, whether or not the envelope protein was present, consistent with the previous results showing that 4E10 binds cell membranes, but not the untriggered envelope spikes (2). Addition of soluble CD4, which induced formation of the CD4i epitope (Fig. S8), did not lead to significant exposure or formation of the 4E10 epitope, suggesting that coreceptor binding is probably needed to induce the prehairpin intermediate conformation of gp41. In short, like 4E10, but unlike VRC01, 10E8 did not interact with the functional, cell-surface expressed envelope trimers, with or without addition of soluble CD4, indicating that the prefusion conformational state of the envelope is not the antibody target. The most striking difference between 10E8 and 4E10 is that the former shows much weaker binding to the 293T cell membrane, which has a
distinct lipid composition from that of HIV-1 membrane (4). Tighter association with cellular membranes by 4E10 might hinder its ability to locate the transient, fusion-intermediate state of gp41; the preference of 10E8 for cholesterol-rich, HIV-1-like membranes and a weaker association with cellular membranes might account for its greater potency.

If 10E8 does not bind native envelope spikes, what then is its neutralization target? We tested binding of 10E8 with our stringently-characterized recombinant proteins in three distinct conformational states: for the prefusion conformation, a stable, homogenous 92UG037.8 gp140 trimer (13, 22); for the prehairpin intermediate state, our construct called “gp41-inter” (12, 13); for the postfusion conformation, a construct termed “gp41-post”, which contains the entire MPER, the transmembrane segment and the fusion peptide in complex with a six helix bundle-specific monoclonal antibody 1281 (Fig. S9; ref (12)). As shown in Fig. 5A and Table S2, 10E8 bound with high affinity (Kd=1.85 nM) to gp41-inter and with lower affinity (Kd=11.7 nM) to the gp41-post-1281 complex in the presence of detergent, which was required to solubilize the gp41-post construct (because of the hydrophobic fusion peptide and transmembrane segment). Under the same conditions, 10E8 had almost undetectable binding to the gp140 trimer, consistent with our FACS data. Since the SPR response is proportional to the molecular mass of binding analyte, the differences between gp41-inter and gp140 are much greater than those shown in the figure. Thus, the binding profile of these preparations is almost the same as that of 4E10: high affinity (Kd=2.34 nM) for gp41-inter and somewhat lower affinity (Kd=7.69 nM) for the gp41-post-1281 complex, but barely detectable binding to gp140 (Fig. 5B). The similarity suggests that these two antibodies neutralize by a common mechanism. Under conditions without detergent, 10E8 again bound strongly to gp41-inter (Kd=0.05 nM), but not to the gp140 trimer (Fig. 5C). Mutant W100bA still had relatively high affinity for gp41-inter (Kd=0.23 nM), but mutants F100aA, F100aA-W100bA and P100fA bound much more weakly. In particular, binding of the P100fA mutant (Kd = 1.23 µM; Fig. S10 and Table S2) was over three orders of magnitude weaker than that of W100aA. As controls, we showed that our gp140 trimer had high affinity for the two anti-gp120 bNAbs, 2G12 (Kd=10.2 nM) and VRC01 (Kd=0.75 nM)
We therefore conclude that 10E8, like 4E10, neutralizes by targeting the fusion intermediate conformational state of gp41.

In a luciferase-based HIV-1 neutralization assay (Table S1), mutants F100aA and W100bA both had a much lower potency than wildtype 10E8. P100fA was only slightly less potent than wildtype 10E8, but much more potent than W100bA despite a much lower affinity for its gp41 target, in agreement with data reported previously (19). Thus, gp41 binding alone cannot account for neutralization potency, further suggesting that loss of membrane binding has a greater impact on neutralization by 10E8 than loss in affinity for gp41.

Improving neutralization potency of 4E10

Antibodies 4E10 and 10E8 both neutralize by targeting the same region of gp41. Although 4E10 binds the gp41 intermediate at least as tightly as does 10E8, it is a much less effective neutralizer. The comparison suggests that altering the lipid binding properties of 4E10 might improve its neutralization potency. We therefore tested whether we could increase 4E10 potency by manipulating its lipid binding, CDR H3 loop. We made several 4E10 Fab variants with single mutations in the CDR H3 to alter its hydrophobicity or structural flexibility (Fig. S11). Although many of these mutations led to reduced or unchanged neutralization, we found one variant, with the mutation G100dA, designed to rigidify the CDR H3, that was consistently more potent at neutralizing all selected viruses (Table 1). Its binding to gp41-inter was even slightly reduced (Figs. S11). Thus, optimization of the membrane-interacting CDR H3 of 4E10 may lead to generation of a 10E8-like neutralizing antibody. The result suggests that we could consider 10E8 as simply a more potent version of 4E10-like antibody.

Discussion

Our results indicate that 10E8 is not an “outlier” among MPER-specific bNAbs, but rather a 4E10-like antibody with greater neutralization potency. We suggest that most MPER bNAbs, if not all, are likely to neutralize by a common mechanism - targeting the
fusion intermediate state of gp41 with the help of their lipid binding activity. The greater neutralization potency of 10E8, when compared to 4E10, highlights the importance of optimal binding to HIV-1 viral membrane and low levels of binding to host cellular membranes. 4E10 is a weaker neutralizer, despite its higher affinity for gp41, probably because of its relatively strong, non-selective binding to lipid bilayers and in particular to the surfaces of cellular membranes (Fig. 4). Tight binding to the cell membrane could diminish its capacity to capture a transient gp41 target, even though some association with the viral membrane is needed to create proximity.

There has been considerable debate about how the MPER-specific antibodies neutralize. Ruprecht and colleagues reported that irreversible HIV-1 neutralization by 2F5 and 4E10 might involve gp120 shedding when the virus was exposed to the antibodies in the absence of target cells (33). In this case, virion neutralization was found to be a very slow process that required several hours. Neutralization in the presence of target cells by these antibodies occurs much more rapidly (well within an hour) (10, 19), however, suggesting gp120 shedding is unlikely to be their primary mode of neutralization. Without receptor engagement, it is conceivable that a small population of gp41 can sample the prehairpin intermediate conformation, which could be captured by 2F5 or 4E10 over a long period of incubation time. Subsequently, stabilization of the intermediate state by the antibodies could lead to gp120 dissociation. It would be interesting to test whether 10E8 is more or less potent than 2F5 or 4E10 under these conditions. Reinherz et al. have proposed yet another mechanism of neutralization based on studies using short, monomeric MPER peptides embedded in lipid bilayers. They concluded that 2F5 and 4E10 might need to extract their core epitopes from the viral membrane in order to form a high affinity complex with gp41 to inhibit viral entry (21, 37). It is unclear what conformational state of gp41 these short peptides represent and which step during the viral fusion process these antibodies would block. Moreover, 10E8 has a lower affinity for gp41 and shows weaker binding to membrane than 4E10. As it would presumably be less effective in extracting its epitope from the membrane, why would it be much more potent than 4E10 in neutralization? Finally, several gp140 preparations have been reported to bind 4E10 or 2F5 (1, 39). Many preparations of envelope trimers or oligomers used in previous studies
have “monomeric gp120-like” characteristics -- for example, binding to CD4-induced antibodies in the absence of CD4 and exhibiting high affinity with non-neutralizing CD4 binding site antibodies. They also either aggregate or dissociate into dimers and monomers during expression or purification and probably fail to mimic the native envelope spikes. In contrast, our stable, homogenous gp140 trimers, despite lack of the cleavage between gp120 and gp41, show a number of properties expected for a native, functional envelope trimer, but only bind 4E10 or 2F5 when denatured in a western blot (13, 22). We demonstrate here that our gp140 trimer also fails to bind native 10E8, fully consistent with the characteristic of the functional envelope trimers expressed on cell surfaces (Fig. 4).

Biochemical studies of protein-membrane interaction are technically challenging. Huang and colleagues did not detect phospholipid binding by 10E8 using SPR with a L1 sensor chip (19). We found that relatively high levels of background binding of hydrophobic proteins to the lipophilic alkyl chains, which capture liposomes on the L1 chip, as detected in the control channel in the absence of detergent, could mask weak signals. Additionally, compositional preference for lipids may also account for lack of binding to certain liposomes by 10E8. We chose for that reason to use three independent approaches to demonstrate lipid binding by 10E8. In particular, for our SPR experiments, we used a streptavidin-coated sensor chip (SA chip) to capture biotinylated liposomes; this sensor chip showed almost no background binding of these antibodies in the absence of detergent. The liposome flotation assay detects the amount of protein-liposome complexes that survive three-hour centrifugation. It is therefore not surprising that the signals for both 4E10 and 10E8 binding to membrane are low because of their relatively fast dissociation rate. Dense precipitates due to crosslinking were clearly visible when IgG of 10E8 or 4E10 was first mixed with liposomes, thereby ruling out the possibility that only a small population of the antibody can bind the liposomes.

Our findings that 10E8 and other MPER-antibodies neutralize by a common mechanism may guide MPER-based vaccine development and help avoid potentially wasteful efforts to chase elusive immunogens that may not be meaningful. These results emphasize the
need for an immunogen with two components: a protein to present the relevant gp41 structure, such as gp41-inter, and lipids to select for lipid binding. In our study, the postfusion gp41 construct containing the intact MPER shows relatively high affinity binding to both 10E8 and 4E10. Because of its stability, it might be a better vaccine antigen than those studied so far (8, 26, 29). Buzon and colleagues reported that 4E10 did not interact with gp41 including the MPER in the post fusion conformation, in agreement with clashes revealed by the molecular docking (5). It is possible that the presence of the fusion peptide and transmembrane segment in our gp41 construct has some impact on the local conformation of the 4E10 epitope, as we found both 4E10 and 10E8 also show binding to our gp41 protein expressed on the insect cell surfaces (data not shown).

Likewise, liposomes with a cholesterol-rich, HIV-1-like lipid composition might be more effective for inducing potent MPER-specific bNAbs than any previously tested, lipid-based immunogens (9, 14, 26). We note that a single chain antibody (VHH), 2H10, recently isolated from llamas immunized with a liposome-based gp41-MPER immunogen, requires a hydrophobic CDR3 for neutralization but not for antigen recognition (26). The mode of neutralization used by this vaccination-induced antibody appears to be consistent with the general mechanism of HIV-1 neutralization by MPER-specific antibodies we propose here.
Acknowledgments

We thank Stephen Harrison, Joanna Magnar, Marcelo Beradi, Eric Salgado, Andrea Carfi and Kurt Swanson for generous advice and assistance. 10E8 expression constructs were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 anti-gp41 mAb (10E8), from Dr. Mark Connors.

We acknowledge support from NIH grants GM083680 (to B.C.), AI084794 (to B.C. and Dan H. Barouch), Collaboration for AIDS Vaccine Discovery (CAVD) grants (to Dan H. Barouch and to Barton F. Haynes) from the Bill and Melinda Gates Foundation, the Center for HIV/AIDS Vaccine Immunology (to Barton F. Haynes). G.F. is partly supported by a Scholar Award from Harvard University Center for AIDS Research.
References:


Figure Legends

FIG. 1. Interactions of 10E8 with lipid bilayers. Liposome flotation assay to detect binding to HIV-1-like membranes at 37°C by various antibodies, including 17b, 4E10, 10E8, 10E8-F100aA and 10E8-W100bA. Seven fractions throughout the sucrose gradient (from top (t) to the bottom (b)) after centrifugation were collected and analyzed by western blot. Heavy and light chains of antibodies are indicated by H and L, respectively.

FIG. 2. Interactions of 10E8 with lipid bilayers. Dynamic light scattering analysis to monitor crosslinking of liposomes with a HIV-1-like lipid composition at 37°C by various antibodies, including 17b, 4E10, 10E8, 10E8-F100aA and 10E8-W100bA. Phosphate buffered saline (PBS) was used as a negative control. Regularization histograms plotting percent of scattering intensity (%intensity) against hydrodynamic radius of particles (Rh) in nanometers are reported.

FIG. 3. Interactions of 10E8 with lipid bilayers. Interactions of lipid bilayers with a HIV-1-like lipid composition (A) and phosphatidylcholine (PC):cardiolipin (CL) = 3:1 (B) with various antibodies were measured by surface plasmon resonance (SPR) at room temperature. Liposomes incorporated with 0.5 % of biotin-labeled phosphatidylethanolamine (PE) were immobilized on the surface of a sensor chip coated with streptavidin and various antibodies, including 17b, VRC01, 4E10, 10E8, 10E8-F100aA, 10E8-W100bA and 10E8-FW-AA (10E8-F100aA-W100bA), at 1 µM were passed over the lipid bilayer surfaces. Recorded sensorgrams are shown in black for 4E10, red for 10E8, purple for 10E8-F100aA, magenta for 10E8-W100bA, dark red for 10E8-F100aA-W100bA, gray for VRC01 and blue for 17b.

FIG. 4. 10E8 targets the prehairpin intermediate conformation of HIV-1 gp41. A. Antibody binding to the HIV-1 92UG037.8 envelope trimer expressed on 293T cell surfaces by fluorescence-activated cell sorting (FACS) analysis. 293T cells were transfected with either 92UG037.8 gp160, or no DNA as a negative control. Binding of antibodies, VRC01, 4E10, 10E8, 4E10+ 2D CD4, 10E8+2D CD4, 10E8-F100aA, 10E8-W100bA and 10E8-F100aA-W100bA, to the cell surfaces were detected by a phycoerythrin-conjugated goat anti-human secondary antibody. Histograms plotting cell...
counts against fluorescence intensity are shown. The vertical dash line in red indicates the
peak center of the untreated 293T cells as a negative control. The experiments were
repeated three times with similar results.

FIG. 5. 10E8 targets the prehairpin intermediate conformation of HIV-1 gp41. A. 10E8
was analyzed by a surface plasmon resonance (SPR) assay for binding to HIV-1 gp140,
gp41-inter, and gp41-post in complex with an antibody 1281 in the presence of 0.1%
dodecyl maltoside. 10E8 IgG was captured to the surface of a sensor chip coated with
Protein A to avoid potential artifacts introduced by protein immobilization. Each of
gp140, gp41-inter or gp41-post/1281 at various concentrations were passed over the
antibody surface individually without regeneration for single cycle kinetic analysis. The
recorded sensogram for gp41-post/1281 is in cyan, gp41-inter in red and gp140 in blue;
and fits in green. The molecular mass of the three envelope constructs are also indicated.
Since the SPR response is proportional to the molecular mass of binding analyte, the
differences between gp41-inter (red) and gp140 (blue) are much greater than those shown
in the figure. B. Similar to b, 4E10 was analyzed for binding to HIV-1 gp140 (blue),
gp41-inter (red), and the gp41-post in complex with anti-gp41 antibody 1281 (cyan). C.
Binding of 10E8 (red) and its variants 10E8-F100aA (purple), 10E8-W100bA (magenta)
and 10E8-F100aA-W100bA (dark red) to gp41-inter was compared by single cycle
kinetic analysis in the standard HBS buffer. Binding of 10E8 to gp140 (blue) was also
performed to evaluate the interaction without dodecyl maltoside. D. Control experiments
by single cycle kinetic analysis to demonstrate binding of gp140 used in A, B and C to
gp120-specific antibodies 2G12 (black) and VRC01 (grey). All binding constructs are
summarized in Table S2.
Table 1. Improved neutralization potency of 4E10 by modifying its lipid-interacting CDR H3 loop.

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