Interactions between Lipids and Human Anti-HIV Antibody 4E10 Can Be Reduced without Ablating Neutralizing Activity[†]

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Human 4E10 is one of the broadest-specificity, HIV-1-neutralizing monoclonal antibodies known, recognizing a membrane-proximal linear epitope on gp41. The lipid cross-reactivity of 4E10 has been alternately suggested either to contribute to the apparent rarity of 4E10-like antibody responses in HIV infections, through elimination by B-cell tolerance mechanisms to self-antigens, or to contribute to neutralization potency by virus-specific membrane binding outside of the membrane-proximal external region (MPER). To investigate how 4E10 interacts with membrane and protein components, and whether such interactions contribute to neutralization mechanisms, we introduced two mutations into 4E10 Fv constructs, Trp to Ala at position 100 in the heavy chain [W(H100)A] and Gly to Glu at position 50 in the light chain [G(L50)E], selected to disrupt potential lipid interactions via different mechanisms. Wild-type and mutant Fvs all bound with the same affinity to peptides and monomeric and trimeric gp140s, but the affinities for gp140s were uniformly 10-fold weaker than to peptides. 4E10 Fv binding responses to liposomes in the presence or absence of MPER peptides were weak in absolute terms, consistent with prior observations, and both mutations attenuated interactions even further, as predicted. The W(H100)A mutation reduced neutralization efficiency against four HIV-1 isolates, but the G(L50)E mutation increased potency across the same panel. Electron paramagnetic resonance experiments showed that the W(H100)A mutation, but not the G(L50)E mutation, reduced the ability of 4E10 to extract MPER peptides from membranes. These results show that 4E10 nonspecific membrane binding is separable from neutralization, which is achieved through specific peptide/lipid orientation changes.

Few of the hundreds of known neutralizing anti-HIV monoclonal antibodies (MAbs) display broad cross-reactive activities (4). Of those derived from clade B-infected patients, b12 binds to the gp120 subunit of the HIV envelope protein (Env), to an epitope that overlaps the CD4 binding site, and neutralizes approximately 50% of virus isolates tested, including non-clade B viruses (27). 2G12 binds to N-linked carbohydrates on gp120 (32, 34) and neutralizes 41% of isolates tested, although not clade C or E isolates. 447-52D also binds to the gp120 subunit, to an epitope within the V3 loop, and potentially neutralizes up to 45% of clade B isolates but rarely non-clade B isolates. 4E10 and 2F5 recognize adjacent epitopes located at the membrane-proximal external region (MPER) of the gp41 Env subunit (9, 22, 24, 28, 42). Two neutralizing antibodies (NAbs) isolated from a clade A-infected patient (PG9 and PG16) show broad and potent neutralizing activity by recognizing epitopes consisting of conserved regions of the V2 and V3 loops of gp120, preferentially on native trimers (40).

4E10 is capable of neutralizing all isolates tested at some level (4), although there is evidence for the existence of rare

viruses that are resistant to 4E10 neutralization (30). The exact structure of the epitope recognized by 4E10 within the trimeric, functional HIV Env is unknown, but structural studies have shown that an isolated peptide spanning the epitope adopts a helical conformation, a short 3₁₀ segment followed by a 4₁₃ (or true α -helical) segment, with an extended structure at the N terminus when bound to 4E10 (9). It has also been reported that 4E10 interacts with a variety of lipids and membrane components, particularly the phospholipid cardiolipin (15), suggesting that difficulties in eliciting 4E10-like broadly neutralizing antibodies by immunization and the apparent rarity of 4E10-like antibody responses in HIV-1-infected subjects (19, 33) are linked to this polyspecificity to autoantigens, contributing to their elimination through tolerance mechanisms. However, subsequent studies have shown that the measurable, but quite weak, affinity of 4E10 for certain lipids is comparable to that of some antiphospholipid antibodies elicited during many infections, suggesting that 4E10 is not remarkably auto-reactive (35). Therefore, it is still unclear whether lipid binding properties are linked to the rarity of 4E10-like specificities. It has also been proposed that the neutralizing activity of 4E10 may partly depend on lipid binding, either through interactions with viral membrane lipids that disturb the membrane-bound structure of the MPER on the trimeric, virion-associated Env spike (39) or through an encounter model. In the latter, initial interactions with membrane components align 4E10 with its

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FIG. 1. 4E10 Fv monobody-diabody equilibration. Results of the SEC analysis of the monobody-diabody equilibrium of scFv4E10 are shown. Freshly separated monobody (A) or diabody (B) preparations of scFv4E10 and cleaved Fv4E10wt (C) were incubated for the indicated time at 4°C (black, initial preparation; yellow, 1 day; orange, 4 days; red, 5 days; green, 8 days; blue, 11 days; purple, 13 days) and then reanalyzed by SEC using a Superdex 75 10/30 column. Peaks are labeled by corresponding molecular species.

protein epitope or allow 4E10 to gain proximity to its epitope (1), perhaps partially alleviating steric occlusion effects (for example, see reference 17). We sought to determine whether specific interactions exist between 4E10 and membrane lipid components and whether such interactions meaningfully contribute to neutralization by any mechanism.

MATERIALS AND METHODS

Cloning, expression, purification, and characterization of engineered proteins. The DNA encoding the variable light and heavy (V_L and V_H) domains of antibody 4E10, joined through a noncleavable 15-mer linker (GGGGSGGGGS GGGGS; the kind gift of Pamela Bjorkman, Caltech), was subcloned into the pET22b vector (Invitrogen) in order to generate a single-chain Fv (scFv) construct of 4E10 incorporating thrombin cleavage sites (LVPR/GS) to eliminate monobody/diabody equilibration (Fig. 1). The linker sequence was changed to LVPRGSGGGGLVPRGS, and the W(H100)A and G(L50)E mutations (Fig. 2) were introduced into this construct by QuikChange mutagenesis (Stratagene) following the manufacturer's protocols.

4E10 constructs were all expressed in *Escherichia coli* BL21(DE3) RIL cells (Invitrogen) as inclusion bodies, solubilized, and denatured in 8 M urea and refolded *in vitro* by following established protocols (38). Refolded proteins were then purified by Ni-nitrilotriacetic acid (Superflow NTA; Qiagen) affinity chromatography, dialyzed into thrombin digestion buffer (20 mM Tris-HCl, pH 8.4,

150 mM NaCl, 2.5 mM CaCl_2), and digested overnight with biotinylated thrombin (Novagen) at 0.05 units per mg of protein at room temperature to generate cleaved Fvs. After complete digestion (verified by SDS-PAGE), biotinylated thrombin was removed with streptavidin-agarose (Novagen). Proteins were further purified by size exclusion chromatography (SEC) using Superdex 75 10/30 and Superdex 75 16/60 columns (GE Healthcare Life Sciences) running in PNEA buffer [25 mM piperazine- N,N' -bis(2-ethanesulfonic acid), pH 7.0, 150 mM NaCl, 1 mM EDTA, 0.02% NaN_3]. Protein concentrations were determined by absorbance at 280 nm, with associated errors estimated at less than 2%. All purified Fvs showed the appropriate disulfide bonding state by comparative reducing/nonreducing SDS-PAGE and were monomeric and monodispersed by analytical SEC and dynamic light scattering (Zetasizer Nano-S; Malvern Instruments) (data not shown). Proteins used for epitope and liposome surface plasmon resonance (SPR) binding studies were repurified by SEC in HBS-EP buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% [vol/vol] P-20 surfactant [Biacore AB]) or phosphate-buffered saline (PBS) buffer (10 mM Na_2HPO_4 , 2 mM KH_2PO_4 , 137 mM NaCl, 2.7 mM KCl [pH 7.2]) within 48 h of analysis. Fab447-52D was prepared by digestion with immobilized papain (Pierce) of IgG 447-52D (provided by Pamela Bjorkman, Caltech) and purified using a protein A column (Pierce) and SEC.

An extended peptide (85 residues; M_r , 10,500) containing the core 4E10 epitope sequence was used for cocrystallization (see below); it was expressed in *E. coli* BL21 Star (DE3) cells (Invitrogen) grown for 4 h at 37°C and subsequently overnight at 18°C in ZYP-5052 auto-induction medium plus kanamycin (100 $\mu\text{g}/\text{ml}$). Cells were pelleted, resuspended in standard buffer (50 mM Tris, 500 mM NaCl, 10 mM imidazole, 0.5 mg/ml lysozyme), sonicated on ice, and clarified by centrifugation. The supernatant was then tumbled with nickel-NTA resin (Superflow NTA; Qiagen) for 30 min at 4°C. The resin was rinsed with 10 mM imidazole twice and 20 mM imidazole twice and then eluted with 250 mM imidazole in standard buffer. The eluate was finally purified by SEC on Superdex 75 16/60 columns (GE Healthcare Life Sciences).

Soluble SF162 gp140 constructs were transiently expressed in HEK 293F cells (Invitrogen) and purified by *Galanthus nivalis* lectin affinity chromatography followed by DEAE anion-exchange chromatography (19, 33). SF162 gp140 monomers and trimers were further separated by SEC on Superdex 200 16/60 columns (GE Healthcare Life Sciences) and verified by SDS-PAGE, native PAGE, Western blotting, and enzyme-linked immunosorbent assay (data not shown).

Circular dichroism (CD) spectra were measured on a J-815 spectrometer (Jasco) using a 1-mm-path-length quartz cuvette and sample volumes of 200 μL . Protein was diluted to 20 μM and exchanged into 10 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$, pH 7.0, immediately prior to analysis. Wavelength scans were recorded from 260 to 190 nm, at 25°C and 50°C, at a scanning speed of 10 nm/minute, and with a data pitch of 0.1 nm. The response and the bandwidth were maintained at 8 s and 1 nm, respectively. The wavelength of the maximal difference between CD curves at the two temperatures was selected for T_m determinations: 210 nm for Fv4E10wt, Fv4E10[W(H100)A], and scFv4E10 and 203 nm for Fv4E10[G(L50)E], where the temperature was changed from 4 to 96°C at a slope of 2°C/minute, data pitch of 2°C, and delay time of 10 s, using the same response and bandwidth settings as the wavelength scans (Fig. 3). T_m s were determined by nonlinear least-squares analysis using a linear extrapolation model (Spectra Analysis; Jasco). Thermal stabilities were confirmed by consistent SPR binding responses to chip-coupled SF162 gp140 monomers over 7-h incubations at 37°C (Fig. 3).

FIG. 2. The structure of the 4E10/epitope complex. Shown is a molecular surface representation of the structure of the 4E10/peptide complex (2FX7.pdb), colored by electrostatic potential (red, negative; blue, positive). The peptide is shown as a licorice-stick representation, colored by atom type (gray, carbon; blue, nitrogen; red, oxygen). Features discussed here have been labeled by residue number and include the protruding side chain of W(H100) at the tip of HCDR3, which, along with other residues here, potentially mediates interactions with membrane components; the putative phosphate binding site between the side chains of two lysine residues (H100E and L32); and the position of the residue [G(L50)] mutated to ablate the putative phosphate binding site by replacement with a site-filling glutamate residue.

FIG. 3. 4E10 Fv solution stability. CD melting curves are shown for Fv4E10wt (A), Fv4E10[W(H100)A] (B), Fv4E10[G(L50)E] (C), and scFv4E10 (D) in 10 mM KH₂PO₄/K₂HPO₄ (pH 7.0). Melting curves were obtained by ramping the temperature from 4 to 96°C at a fixed wavelength of 210 nm. Double-reference-subtracted SPR responses are shown for 35 nM Fv4E10wt (E), Fv4E10[W(H100)A] (F), and Fv4E10[G(L50)E] (G) analytes incubated at 37°C and then flowed over immobilized SF162 gp140 monomers every hour for 7 h to model the conditions of extended incubation during the neutralization assays. The curves are shown in a range of colors for the different time points, but all nearly perfectly superimpose. All sample injections, including buffer blank, were carried out at 30 μ l/min for 5 min, followed by a 5-min dissociation phase.

Crystallization and crystallography. Of the possible combinations available, only the complex between Fv4E10[W(H100)A] and an extended peptide with the 4E10 epitope sequence embedded within yielded usable crystals. Initially, diffraction data ($d_{\min}$, 2.9 Å) were collected in house at -170°C from a crystal grown by vapor diffusion ([protein] = 9.6 mg/ml; well solution, 0.2 M magnesium

acetate, 10% polyethylene glycol 8000 [PEG 8000]) on a Saturn 944+ charged-coupled-device detector with HF optics (Rigaku) and processed with d*TREK (29); the crystal was cryo-protected with ethylene glycol. Initial structure factor phases were determined by molecular replacement, using the program Phaser (20) and a search model consisting of the 4E10 variable domains (9). The initial

FIG. 4. Structure of the 4E10 Fv/peptide complex. (A) Cartoon tube representations of superpositions of the backbones of the variable domains of Fab4E10 (from 2FX7.pdb [gray]) and the two molecules (blue or purple) in the asymmetric unit of the crystal structure of Fv4E10[W(H100)A]; V_L domains are on the right, and V_H domains are on the left. The backbone tubes have their diameter scaled by crystallographic B-factor, illustrating relative mobility, and are transparent to show overlap. The position of W(H100) is indicated by the red shading of the Fab4E10 tube. (B) Stereo diagram of licorice-stick representations of the 4E10 epitope peptide from the Fab4E10 complex structure (2FX7.pdb) and two complexes in the asymmetric unit of the crystal structure of Fv4E10[W(H100)A], colored by atom type (carbon, gray; nitrogen, blue; oxygen, red) for residues within the canonical epitope and dark gray for non-epitope residues. Residues have been labeled where possible. (In these studies, the epitope was embedded in a longer, scaffolding sequence as part of an effort to design proteins to display HIV epitopes as potential vaccine candidates [B. Schief and coworkers, unpublished results]; this particular construct did not fold cooperatively into a defined structure and therefore serves as a suitable surrogate for the isolated epitope peptide.) Residues in the core 4E10 epitope have been labeled, where legible.

electron density map showed good density for both Fvs (two complexes in the asymmetric unit) but only for the epitope regions of the extended epitope peptide, which was shown to be disordered in solution by circular dichroism (no evidence for ordered secondary in the spectrum, and the T_m trace is onsigmoidal, indicative of the absence of a cooperative folding transition [data not shown]). Two rounds of modeling and positional and group B factor refinement were carried out with the program Coot (14) and the program package CNS (6).

Subsequently, 2.7-Å resolution diffraction data were collected on a crystal grown under similar conditions (well solution of 0.2 M calcium acetate, 0.1 M HEPES, pH 7.0, 8% PEG 8000). The data were collected in house on an R-AXIS IV++ image plate detector with HR optics (Rigaku) at -170°C and processed with d*TREK; the crystal was cryo-protected by the addition of 70% sucrose. The program Refmac5 (21) was used to carry out positional and individual B factor refinement against this data set, beginning with the lower-resolution model. Partway through refinement, the model was submitted to the TLSMD server (25, 26) and six TLS groups were defined, one per polypeptide chain. The final model (Fig. 4) contains only 18 or 21 (out of 85) residues, comprising the 10-residue epitope and immediately flanking residues, for the two extended peptide chains, as the remainder are too disordered to model.

Structure validation was carried out with Procheck (18), the MolProbity server (11), and the RCSB ADIT validation server. Data collection and structure refinement statistics are shown in Table 1.

SPR analyses of IgG, Fab, and Fv/gp140 or peptide interactions. Protein-protein and protein-peptide interaction analyses by SPR were conducted at 25°C in HBS-EP buffer on a Biacore T100 system. For analysis of the binding of 4E10 Fv and Fab analytes (wild type and mutant) (see Table 2) (Fig. 5) to the peptide corresponding to the 4E10 epitope in clade B R5 HIV-1 SF162 isolate plus four C-terminal lysine residues (NWFDISKWLWYIKKKK) (10), peptide at 10 $\mu\text{g}/\text{ml}$ in 10 mM sodium acetate (pH 5.5) was covalently immobilized on a CM5 research-grade sensor chip (Biacore) by standard amine coupling chemistry following the manufacturer's protocols; reference flow cells were left blank. The C-terminal lysines were added to aid coupling and match peptides used in prior studies (8). Immobilization of 240 response units (RU) resulted in optimal responses in subsequent analyses. Different concentrations of Fv/Fab 4E10 analytes were injected in randomized duplicate runs, at a flow rate of 10 $\mu\text{l}/\text{minute}$ for 35 min to reach equilibrium, followed by a 10-min dissociation phase. Optimal regeneration was achieved by injection of 10 mM glycine (pH 1.5) at a flow rate of 20 $\mu\text{l}/\text{minute}$ for 6 s followed by HBS-EP buffer stabilization for 5 min. For binding analysis of Fab/Fv 4E10s and Fab447 analytes to soluble SF162 gp140 constructs (see Table 2) (Fig. 6), 10 $\mu\text{g}/\text{ml}$ SF162 gp140 monomer or trimer in 10 mM sodium acetate (pH 5.0) was immobilized on a CM5 chip by direct amine coupling chemistry. Reference flow cells were likewise left blank. Immobilization of a 940-RU monomer and 1,330-RU trimer resulted in optimal responses for subsequent kinetic analyses, resulting in surfaces with specific activities estimated at approximately 20% and 30%, respectively. All sample injections were carried out in randomized duplicate runs, at 50 $\mu\text{l}/\text{minute}$ for 6 or 7 min for Fab/Fv 4E10s followed by a 5-min dissociation period and at 50 $\mu\text{l}/\text{minute}$ for 3 min for Fab447 followed by a 3-min dissociation period. Regeneration was performed as described above.

SPR binding studies of IgG447 to the peptide corresponding to the 447 epitope in clade B HIV isolate SF162 (KSITIGPGRAKKKK) (see Table 2) (Fig. 7) were conducted at 25°C in HBS-EP buffer with 0.1 mg/ml bovine serum albumin and 1 mg/ml CM-dextran on a Biacore 3000 system (Biacore AB). Goat anti-human IgG Fc γ fragment-specific antibody (Jackson ImmunoResearch), at 30 $\mu\text{g}/\text{ml}$ in 10 mM sodium acetate (pH 5.5), was immobilized at a density of 14,000 RUs on two flow cells of a CM5 research-grade sensor chip (Biacore) by standard amine coupling chemistry. The 447 IgG at 5 $\mu\text{g}/\text{ml}$ was captured on one flow cell of immobilized anti-Fc Ab at a flow rate of 10 $\mu\text{l}/\text{minute}$ for 5 min to reach a 1,300-RU response, leaving the other immobilized anti-Fc antibody surface as the reference. Different concentrations of SF162 447 peptide (25 nM to 6 μM by 3-fold dilutions, including buffer as blank) were injected in randomized triplicate runs at a flow rate of 50 $\mu\text{l}/\text{minute}$ for 3 min to reach equilibrium, followed by a 3-min dissociation phase and a 30-s wash with running buffer.

Sensorgrams obtained from kinetic SPR measurements were analyzed by the double subtraction method described by Myszk (23). The signal from the reference flow cell was subtracted from the analyte binding response obtained from flow cells with immobilized ligands. Buffer reference responses were then averaged from multiple injections. The averaged buffer reference responses were then subtracted from analyte binding responses, and the final double-referenced data were analyzed with BIAevaluation 2.0 or 4.1 software (Biacore), globally fitting data to derive kinetic and equilibrium parameters. Sensorgrams for binding of Fab/Fv 4E10s with gp140 constructs were fitted using a simple one-to-one binding model (Fig. 6A to H), while sensorgrams for binding of Fab447 with

TABLE 1. Crystallographic statistics for the Fv4E10[W(H100)A] epitope/peptide complex structure

Parameter	Value ^a
Data collection	
Space group	P6 ₅ 22
Cell dimensions (Å).....	a = b = 92.4, c = 272.8
Resolution (Å).....	69.02–2.70 (2.80–2.70)
No. of observed reflections.....	89,106 (8,305)
No. of unique reflections	19,557 (1,937)
Redundancy	4.56 (4.29)
Completeness (%).....	98.6 (99.6)
R_{merge}	0.104 (0.347)
Avg $I/\sigma(I)$	7.5 (2.9)
Structure refinement	
Resolution (Å).....	60.08–2.70
$R_{\text{work}}/R_{\text{free}}$	0.229/0.287
No. of atoms	
Protein	3,794
Water	55
Other (Ca ²⁺)	1
RMSD from ideal values	
Bond lengths (Å).....	0.008
Bond angles ($^\circ$).....	1.109
Chiral vol (Å ³).....	0.061
Ramachandran plot statistics (Procheck)	
Residues in most favored regions (%).....	88.0
Residues in additional allowed regions (%).....	11.5
Residues in generously allowed regions (%).....	0.0
Residues in disallowed regions (%).....	0.5 (2 residues ^b)
Est. coordinate error (max. likelihood ESU ^c) (Å).....	0.26
Avg B-factor (molecule A/molecule B)	
Fv light chains.....	68.8/59.5
Fv heavy chains	53.7/52.9
Epitope scaffolds	69.0/70.7
Waters.....	47.3

^a Values in parentheses are for the highest-resolution shell.

^b Fv light chain residue 52 (51 by Kabat numbering) is in a highly conserved γ -turn.

^c ESU, estimated standard uncertainty.

gp140 constructs were fitted by a two-state reaction model (Fig. 6I and J), which assumes 1:1 binding of analyte to immobilized ligand followed by a conformational change that stabilizes the complex. However, it is important not to over-interpret use of one interaction model over another; a better fit may be achieved simply by additional parameters and may not reflect fundamentals of the system. For sensorgrams obtained from equilibrium measurements of Fab/Fv 4E10s with 4E10 peptide and IgG447 with 447 peptide, steady-state binding levels of analytes were plotted against analyte concentrations, from which an equilibrium constant was estimated by using BIAevaluation 2.0 and 4.1 software (Biacore), respectively (Fig. 5 and 7).

ITC analyses. The binding of the SF162 4E10 epitope peptide (NWFDISKWLWYIKKKK) to intact IgG 4E10 was examined by isothermal titration calorimetry (ITC) on a VP-ITC microcalorimeter (MicroCal) at 25°C following the manufacturer's guidelines (Fig. 8). All samples were dialyzed into PNEA buffer at pH 7.0, filtered, and degassed immediately before use; the reference cell contained degassed water. 4E10 IgG (at 1.5 μM) was used as titrand, and epitope peptide (at 35 μM) was used as titrant. Each experiment consisted of 40 injections (7 μl each) at 240-s intervals at a stirring speed of 329 rpm. Following integration and normalization of the peaks, data were fit using Origin 7 SR2 software (OriginLab). Baselines were manually adjusted as needed, and data were modeled using the one-site binding algorithm.

SPR analyses of protein-liposome interactions. Binding of Fvs to liposomes in the presence or absence of MPER peptides by SPR (Fig. 9) was performed as

FIG. 5. SPR analysis of 4E10 Fab- and Fv-peptide interactions. Equilibrium SPR measurements of 4E10 Fab and Fv analytes binding to chip-coupled SF162 4E10 epitope peptides are shown. All sample injections were carried out in a randomized duplicate run, at 10 μ l/min for 35 min to reach equilibrium, followed by a 10-min dissociation phase. Double-reference-subtracted SPR responses are shown in panels A, C, E, and G, and corresponding binding analyses are shown in panels B, D, F, and H: Fab4E10 (A and B); Fv4E10wt (C and D); Fv4E10[W(H100)A] (E and F); Fv4E10[G(L50)E] (G and H).

previously described (39); briefly, 1 μ M Fv analytes was flowed over liposomes or liposome-embedded MPER peptides (wild-type [residues 662 through 683; EL DKWASLWNWENITNWLWYIK] or incorporating point mutations) bound on L1 sensor chips (Biacore) for 3 min at a flow rate of 5 μ l/minute.

In vitro neutralization assays. Neutralization assays (see Table 3, below) were performed using single-round entry-competent viruses and TZM-bl cells as targets, as previously described (13). A predetermined amount of virus was mixed or not with serially diluted MAb, Fab, or Fv at 37°C for 1.5 h. Fifty microliters

FIG. 6. SPR analysis of 4E10 Fab and Fv, or 447-52D Fab, interactions with SF162 gp140s. SPR measurements are shown for the binding kinetics of 4E10 Fab (A and E) and Fv (B, C, D, F, G, and H) or 447-52D Fab (I and J) analytes interacting with SF162 gp140 monomers (A, B, C, D, and I) or trimers (E, F, G, H, and J); all sample injections were carried out in randomized duplicate runs at 50 μ l/min. Double-reference-subtracted SPR responses (black), with the corresponding kinetic fits (red), for five analyte concentrations are shown for the following: Fab4E10/gp140 monomer (A); Fv4E10wt/gp140 monomer (B); Fv4E10[W(H100)A]/gp140 monomer (C); Fv4E10[G(L50)E]/gp140 monomer (D); Fab4E10/gp140 trimer (E); Fv4E10wt/gp140 trimer (F); Fv4E10[W(H100)A]/gp140 trimer (G); Fv4E10[G(L50)E]/gp140 trimer (H); Fab447 versus monomeric gp140 (I); Fab447 versus trimeric gp140 (J). Data were analyzed with BIAevaluation 2.0 software (Biacore) and globally fit to derive kinetic and equilibrium parameters using a simple one-to-one binding model for 4E10 Fab and Fv analyses or using a two-state reaction model, which describes a 1:1 binding of analyte to immobilized ligand followed by a conformational change that stabilizes the complex, for 447 Fab analyses.

FIG. 7. SPR analysis of 447-52D IgG-peptide interactions. Equilibrium SPR measurements of SF162 447 epitope peptide analyte binding to captured IgG447 are shown. All randomized, triplicate sample injections were run at 50 μ l/min for 3 min to reach equilibrium, followed by a 3-min dissociation phase. (A) Double-reference-subtracted SPR responses; (B) the corresponding binding analysis.

of the virus-antibody mixture was added to wells of flat-bottom 96-well tissue culture plates containing 3,000 Polybrene-treated TZM-bl cells and incubated for 3 days at 37°C. The cell supernatant was then removed and 100 μ l of SteadyLite Plus (Perkin-Elmer) was added to each well and allowed to incubate for 15 min at room temperature. The number of relative light units associated with 75 μ l of cell lysate was determined on a Fluoroskan Ascent FL fluorometer (Thermo Labsystems). Percent neutralizations at each antibody concentration were calculated as previously described (13), and the 50% inhibitory concentrations (IC_{50} s) were determined by nonlinear regression (one-phase exponential association) using Prism (GraphPad).

EPR spectroscopy. The electron paramagnetic resonance (EPR) experiments (Fig. 10) were performed as previously described (39). The HIV-1 MPER segment (residues 662 through 683; ELDKWASLWNWFDITNWLWYIK) containing a single cysteine substitution at residues L669, W670, I675, W678, and Y681 was synthesized at the Tufts Peptide Synthesis Core Facility (Boston, MA). Peptides were spin labeled with (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl)-methanethiosulfonate (MTSL; Toronto Research Chemicals, Ontario, Canada) and subsequently purified and separated from free spin-labels by reverse-phase high-pressure liquid chromatography as described in reference 39. EPR spectra were recorded on a Bruker EMX spectrometer at 2-mW incident microwave power with a field modulation of 2.0 G at 100 kHz using a Bruker high-sensitivity resonator. Power saturation measurements were performed on a loop-gap resonator with microwave power from 0.4 to 100 mW, and samples were purged by

a stream of either air or nitrogen gas. The immersion depth values were calculated based on the ratio of the accessibility value of O_2 to 50 mM nickel(II) ethylenediaminediacetic acid (NiEDDA).

Protein sequence accession number. The protein structure for the 4E10 Fv/peptide complex has been deposited in the RCSB Protein Data Bank (2) and assigned accession code 3H3P.

RESULTS

Strategy. The structure of Mab 4E10 (Fig. 2) reveals several conspicuous features outside of the interface with a peptide spanning its linear epitope: a prominently protuberant tryptophan side chain (H100) at the apex of HCDR3 and a positively charged pocket on the light chain. Both features were hypothesized to mediate interactions with viral membrane components, the latter by possibly binding, specifically or nonspecifically, to negatively charged phospholipid head groups. Two mutations were designed to test the role of these structural features on neutralization and ligand interactions: W(H100)A, which eliminates the side chain, and G(L50)E, which fills the

FIG. 8. ITC analysis of 4E10 IgG-peptide interactions. ITC measurements of the binding of IgG4E10wt (A) and IgG4E10[G(L50)E] (B) to SF162 epitope peptides at 25°C are shown. Both raw ITC isotherms (top panels) and data after integration and normalization (bottom panels) are shown. The solid lines (red) in the bottom panels show the fits with a one-site binding model.

FIG. 9. Binding of 4E10 to liposomes with or without MPER peptides. Qualitative SPR responses, as described in reference 39, are shown for Fv4E10wt, Fv4E10[W(H100)A], and Fv4E10[G(L50)E] analytes (at 1 μ M) interacting with chip-bound, HIV virion mimic liposomes (A) or virion mimic liposomes plus wild-type MPER peptide (B), a Y681A mutant MPER peptide (C), or an I682A mutant peptide (D).

positively charged pocket with a negatively charged glutamate side chain. Neither mutation was predicted to perturb protein-epitope interactions, and both were introduced into various 4E10 constructs and tested in binding and neutralization assays. There were no other striking features in the 4E10 combining site that would be obvious candidates for mediating hypothetical interactions with membrane components independent of interactions with the protein epitope. Consequently, our studies focused on these two residues.

4E10 Fvs recapitulate the behavior of 4E10 Fab fragments. SEC analysis of the intact scFv 4E10 construct (Fig. 1) clearly showed dynamic equilibration of monobody and diabody forms (16). Since such behavior complicates interaction analyses (and possibly crystallization), we engineered cleavable Fv constructs {Fv4E10wt, Fv4E10[W(H100)A], and Fv4E10[G(L50)E]} that preclude diabody formation. After protease treatment, these constructs are stably monodispersed in solution, running exclusively as monomers by SEC (Fig. 1), with no apparent proteolysis observed even after extended storage. All 4E10 Fvs exhibited exquisite solution stability, with CD T_m values ranging narrowly from 57.0 to 58.9°C for cleaved Fv and scFv constructs, mutant or wild type (Fig. 3), and consistent SPR binding responses to chip-coupled SF162 gp140 monomers, even over 7-h incubations at 37°C (Fig. 3). Structural comparisons between 4E10 Fab/peptide structure 2FX7 and the structure of the complex between Fv4E10[W(H100)A] and the ex-

tended epitope peptide (Table 1) show a high degree of structural conservation, both between Fab and Fv (Fig. 4; pairwise root mean square deviations (RMSDs) on all common C α s ranged between 0.5 and 0.6 Å for Fv/Fv and Fv/Fab superpositions) and in the details of the interaction with epitope, despite significantly different crystallization conditions, space groups, and crystal packing. The conformation of the epitope peptide is essentially identical (Fig. 4), as are contacts with the antibody combining site, in the region conserved between the extended peptide used here and the minimal peptide used in prior studies. Therefore, the use of Fv constructs here as surrogates for Fab or intact antibodies is justified on the basis of multiple criteria.

Binding studies of Fvs to epitope peptides and Env proteins.

The interactions between 4E10 Fvs or 447-52D IgG/Fab and (i) peptides comprising their linear epitopes (Fig. 5 and 7) or (ii) soluble gp140 proteins (Fig. 6) were first evaluated using SPR (Table 2). For 4E10, Fv analytes were flowed over immobilized epitope peptides or monomeric or trimeric gp140, ensuring that univalent affinities were measured instead of polyvalent avidities; likewise, 447-52D epitope peptides were flowed over IgG 447-52D, or Fab 447-52D was flowed over monomeric or trimeric gp140, for comparison. SPR analysis of the binding to epitope peptides showed that Fv4E10wt, Fv4E10[W(H100)A], and Fv4E10[G(L50)E] bind to the SF162 4E10 epitope peptide with the same affinity as

FIG. 10. MPER conformational changes upon Fv4E10 binding as determined by EPR. (A) MPER residue mobility changes upon Fv4E10 binding, as determined by EPR. EPR spectra of R1 side chains in membrane-bound MPER peptides are shown; black traces represent EPR spectra in the absence of Fv4E10s, while red traces represent spectra recorded in the presence of wild-type or mutant Fv4E10s. Arrows indicate examples of features attributed to highly mobile spectra (the black arrow refers to the spectrum of L669 in the absence of Fv) or highly immobile ones (the red arrow refers to the spectrum of I675 in the presence of Fv). (B) Comparison of wild-type and mutant Fv4E10-induced membrane immersion depth changes of MPER residues. Depth values of MPER R1 residues in the unbound state are shown with gray columns; residue immersion depths of MPER peptides in complex with wild-type or mutant Fv4E10s are shown as black columns. Depth values between -5 Å and 0 Å correspond to the lipid head group region, and depth values larger than 0 Å correspond to the acyl chain region. Reference residues L669 and W670 were compared with the other two residues, with Fv4E10[W(H100)A]-induced depth changes highlighted with red boxes.

that of Fab4E10, with equilibrium dissociation constants (K_D) ranging narrowly from 10 to 16 nM, which is consistent with prior measurements (K_D of 20 nM [5]) for the affinity of chip-coupled 4E10 Fab interacting with a peptide corresponding to the clade B consensus sequence of the 4E10 epitope (NWFD ITNWLWYIKKKK) (3) as analyte. This result confirms that Fv retains the affinity of Fab and that neither mutation introduced into the Fv construct affects the interaction with epitope peptides. To complement the SPR analyses, the equilibrium binding constants between 4E10 IgGs and the SF162 epitope peptide were also determined by ITC (Fig. 8), which additionally measures binding thermodynamics and stoichiometry. The data were best fit by a one-site binding model, giving stoichiometries (N) of 2.05 ± 0.01 (mean $\pm$ standard deviation) for IgG4E10wt and 2.17 ± 0.01 for IgG4E10 [G(L50)E], consistent with the binding of two peptides per IgG (attempts to express IgG4E10[W(H100)A] for comparison here and in subsequent experiments were unsuccessful). The measured equilibrium constants of 4E10 epitope peptide to IgG4E10wt and IgG4E10[G(L50)E] were 53 ± 4 nM and 37 ± 3 nM, respectively, in close agreement with the SPR results. The estimated thermodynamic parameters from this analysis were a ΔH° of -22.6 ± 0.2 kcal/mol and ΔS° of -42.3 cal mol $^{-1}$ K $^{-1}$ for IgG4E10wt and ΔH° of -20.6 ± 0.2 kcal/mol and ΔS° of -35 cal mol $^{-1}$ K $^{-1}$ for IgG4E10[G(L50)E].

SPR binding studies of the interactions between wild-type 4E10 Fab and wild-type and mutant Fvs and SF162 monomeric or trimeric gp140 Env proteins (Table 2 and Fig. 6) again showed that the equilibrium dissociation constants for the three Fvs (wild type, [W(H100)A], and [G(L50)E]) were essentially identical to each other and that, surprisingly, when interacting with either gp140 monomers or trimers, all interactions with gp140 proteins were uniformly approximately 10-fold weaker than those to 4E10 epitope peptides. As a comparison, we also determined the binding of the anti-V3 loop antibody 447-52D to a peptide (the minimal epitope sequence from SF162 plus four C-terminal lysine residues, KSITIGPGRAKKKK) (Table 2 and Fig. 7) and the binding of Fab 447-52D to gp140 monomers or trimers (Table 2 and Fig. 6). While binding by Fab 447 was, overall, weaker than Fab or Fv 4E10, binding to gp140 monomers and trimers was again comparable but 2-fold stronger than to the corresponding epitope peptide.

Binding of Fvs to lipid bilayers. The interactions between Fvs and liposomes were then assayed by SPR, using liposomes

TABLE 2. Binding kinetics of interactions between anti-HIV antibody fragments to SF162-derived epitope peptides^a

Epitope	K_D (nM)	SF162 gp140 monomer binding			SF162 gp140 trimer binding		
		k_{OFF}/k_{ON} (nM)	k_{ON} (M $^{-1}$ s $^{-1}$)	k_{OFF} (s $^{-1}$)	k_{OFF}/k_{ON} (nM)	k_{ON} (M $^{-1}$ s $^{-1}$)	k_{OFF} (s $^{-1}$)
Fv4E10wt	12 (1)	108 (1)	[2.50 (2)] E4	[2.70 (2)] E-3	98 (1)	[2.70 (2)] E4	[2.60 (2)] E-3
Fv4E10[W(H100)A]	14 (1)	106 (2)	[2.40 (2)] E4	[2.50 (2)] E-3	119 (2)	[2.10 (2)] E4	[2.50 (2)] E-3
Fv4E10[G(L50)E]	16 (1)	136 (2)	[2.00 (2)] E4	[2.70 (2)] E-3	114 (2)	[2.00 (2)] E4	[2.30 (2)] E-3
Fab4E10	10 (1)	130 (2)	[1.70 (2)] E4	[2.20 (2)] E-3	135 (2)	[2.00 (2)] E4	[2.70 (1)] E-3
IgG447	199 (6)						
Fab447 (state 1)		92 (2)*	[3.40 (5)] E5	[4.90 (9)] E-2	93 (1)*	[2.60 (2)] E5	[3.60 (3)] E-2
Fab447 (state 2)			[2.06 (2)] E-3	[3.45 (4)] E-3		[2.66 (7)] E-3	[5.8 (1)] E-3

^a Values are means, with standard errors in parentheses. Epitope peptides were the 4E10 or 447-52D sequence or gp140 monomers or trimers as measured by SPR at 25°C. *, equilibrium constants were determined using BIAevaluation 2.0 software (Biacore AB) with a two-state fitting model; state 2 on-rates have units of s $^{-1}$.

TABLE 3. Neutralization IC₅₀s for various 4E10 constructs against four HIV-1 clade B isolates

4E10 construct	Neutralization IC ₅₀ (SE) [relative IC ₅₀] ^a (μM) against HIV isolate:			
	SF162	SS1196	HxB2	JRFL
IgG4E10wt	0.012 (4) [0.09]	0.002 (1) [0.03]	0.005 (1) [0.2]	0.008 (4) [0.3]
IgG4E10[G(L50)E]	0.009 (6) [0.07]	0.0015 (7) [0.02]	0.003 (2) [0.1]	0.004 (2) [0.1]
Fab4E10wt	0.062 (7) [0.5]	0.046 (9) [0.7]	0.007 (4) [0.3]	0.009* [0.3]
Fv4E10wt	0.13 (2) [1]	0.07(1) [1]	0.026(7) [1]	0.029 (3) [1]
Fv4E10[W(H100)A]	>0.8 [>6]	0.22 (2) [3]	0.09 (2) [3]	0.072 (5) [2]
Fv4E10[G(L50)E]	0.1 (2) [0.8]	0.02 (1) [0.3]	0.013 (9) [0.5]	0.0103 (8) [0.4]

^a Relative IC₅₀s, referenced to Fv4E10wt, are calculated in each column separately for each isolate and are shown in brackets. *, performed only in duplicate, and so no error estimate was calculated.

constituted of HIV-1 virion membrane with or without MPER peptides (wild type or incorporating point mutations) and a methodology previously described (39) (Fig. 9). Both mutant Fvs showed reduced binding to liposomes or liposome-embedded MPER peptides relative to the wild type, with the lowest responses alternating between Fv4E10 [W(H100)A] and Fv4E10[G(L50)E].

MPER conformational changes upon Fv4E10 binding. Since the binding of Fv4E10 mutants to liposomes was reduced in both the presence and absence of MPER peptide, we examined whether the reduced binding was solely due to reduced affinity for membrane components or whether the interaction with the peptide was also altered in the context of the membrane. We used EPR to study MPER peptides with side chains (R1) spin labeled at different positions to determine if wild-type or mutant Fv4E10 binding drives conformational changes in the MPER in the membrane-bound state. We first examined the Fv4E10-induced MPER residue mobility changes by comparing the EPR spectra of Fv4E10-bound MPER peptides with that of MPER alone (Fig. 10). Five spin-labeled residues (L669R1, W670R1, I675R1, W678R1, and Y681) previously found to retain Fab 4E10 binding and induce significant spectral changes were chosen to provide appropriate measurements (39). Residues L669 and W670 are located at the N-helix of the MPER, and residues I675, W678, and Y681 are located at the C-helix of the MPER. Consistent with previous findings (39), pronounced EPR spectral changes were observed for all five residues upon wild-type Fv4E10 binding. For these residues, EPR spectra with broader peak-to-peak splitting were found compared to Fv4E10-free MPER side chains, indicating decreased mobility of these R1 residues. Due to the difference of the disposition of each residue at the 4E10 binding pocket, varied spectral changes were observed for these five residues. Notably, comparable EPR spectra changes were found for all five spin-labeled MPER residues upon Fv4E10 [W(H100)A] and Fv4E10[G(L50)E] binding compared to that of wild-type 4E10. These results indicate the presence of very similar or identical MPER conformations at the antibody binding interface for wild-type and mutant Fv4E10s, consistent with the crystallographic results. In conjunction with the comparable SPR affinity measurements, there were no detectable quantitative binding differences by these two methods.

To determine whether the mutant versus wild-type Fv4E10s differentially alter the orientation of the MPER peptide in the membrane upon Fv binding, we measured the membrane immersion depth values of spin-labeled MPER residues in the absence

or presence of Fvs (Fig. 10). IgG4E10wt was previously found to lift the MPER N-helix up out of the membrane, resulting in substantial changes in immersion depth for L669 and W670 with little if any effect on C-helix residues (including I675R1 and W678) (39). Here, Fv4E10wt binding changed the membrane immersion depth comparably to IgG4E10wt; binding of Fv4E10 [G(L50)E] induced depth changes similar to those induced by Fv4E10wt. However, the changes in immersion depth at N-helix residues L669 and W670 induced by Fv4E10[W(H100)A] binding were considerably reduced compared to those induced by Fv4E10wt binding. Fv4E10wt, Fv4E10[W(H100)A], and Fv4E10 [G(L50)E] induced similar immersion depth changes upon binding at MPER C-helix residues I675 and W678. These results suggest that the W(H100)A mutation in Fv4E10 reduces the ability of Fv4E10 to lift the MPER up from the membrane, while G(L50)E retains this capacity.

Neutralization by mutant 4E10 antibodies and Fvs. Finally, the ability of mutant and wild-type Fv4E10 proteins, wild-type Fab4E10, and wild-type and G(L50)E IgG to neutralize a panel of clade B isolates (SF162, SS1196, HxB2, and JRFL) was evaluated (Table 3). Fv4E10[G(L50)E] had comparable or greater neutralization potency to Fv4E10wt across this panel, whereas Fv4E10[W(H100)A] displayed an approximately 2- to greater-than-8-fold reduction in potency (as measured by IC₅₀). The neutralization potency of Fab4E10wt varied from approximate parity to 4-fold greater than that of Fv4E10wt and from approximate parity to 3-fold weaker than Fv4E10[G(L50)E]; the potencies of IgG4E10wt or IgG4E10[G(L50)E], while comparable to each other, were approximately equivalent to or up to 5-fold stronger than Fab4E10wt, varying with isolate, and were either approximately equivalent to or up-to-10-fold stronger than the best Fv potencies. In general, the trend of neutralization potency is IgG4E10wt ~ IgG4E10[G(L50)E] > Fab4E10wt > Fv4E10[G(L50)E] > Fv4E10wt >> Fv4E10[W(H100)A].

DISCUSSION

Approximately 20 to 30% of HIV-1-infected subjects develop broad NAb responses during natural infection (3, 19, 37), so there is hope that an appropriate immunogen can be designed that would elicit comparable or better antibody responses, deemed integral to an effective HIV vaccine. Although not every epitope recognized by naturally generated, broadly reactive NAb is known, a few have been very well characterized (7). MAb 4E10 displays the broadest neutralizing potential of all the currently known broadly reactive NAb

against HIV (4). The epitope of 4E10 is located close to the viral lipid membrane, and there is speculation that neutralization by 4E10 requires binding not only to the HIV Env protein but also to the surrounding viral membrane lipids. To more fully characterize the interactions between 4E10 and its epitope on HIV and to better understand whether lipid binding is important for the broad 4E10 neutralizing potential, we conducted a series of binding studies, focusing on the highly sensitive clade B HIV isolate SF162. Using an Fv construct as a surrogate for intact antibody, we found that Fv4E10 bound (K_D , 12 nM) to a minimal peptide (NWFDISKWLWYIKKK) spanning the linear epitope on SF162 comparably to a corresponding consensus clade B peptide (NWFDITNWLWYIKKKK), despite several nonconservative substitutions in the two sequences. Crystallographic analyses, reported here and previously (9), showed the peptides bind in a structurally conserved manner, consistent with this result, with the conservative S/T substitution likely recapitulating key interactions and the side chain at the K/N position pointing outwards, away from the antibody. Fv4E10 bound comparably to both SF162 gp140 monomers and trimers (K_D , 108 nM and 98 nM, respectively), suggesting that the epitope was presented in a similar manner in both the monomeric and trimeric contexts. This result would be consistent with models where the folding of this section of gp140 is independent of multimerization. Strikingly, however, Fv4E10 bound approximately 10-fold better to the epitope as an isolated peptide. This result was counterintuitive, because there should always be an entropic penalty for binding to a conformationally unconstrained peptide not present when binding to the same sequence structured and ordered by virtue of being embedded in the intact, folded structure of the parent protein. To illustrate the point, we compared the binding of Fab447 to a minimal peptide (K_D , 0.2 μ M) and to gp140 monomers and trimers (K_D , 92 nM or 93 nM, respectively). Here, binding to the peptide was approximately 2-fold weaker than to gp140, as expected, although again, 447 IgG bound comparably to gp140 monomer and trimer. The 4E10 result suggested that this epitope is constrained to adopt a conformation, in the context of gp140, that is significantly different from the ideal binding conformation in the Fab and Fv peptide complex structures and/or that additional steric constraints imposed by gp140, such as glycosylation, partially occlude 4E10 binding.

The absolute values of the interactions between Fv4E10 and lipid bilayers are quite weak, precluding quantitative analysis. However, our data showed that either mutation introduced into Fv4E10, W(H100)A or G(L50)E, measurably reduced interactions with viral membranes. The G(L50)E mutation was designed to ablate a putative phosphate binding site on the surface of the 4E10 combining site that may be able to interact with exposed lipid phosphate head groups independently of 4E10 interactions with its protein epitope. The W(H100)A mutation protrudes from the combining site, reasonably positioned to interact with membrane components when binding Env embedded in viral membranes. We conclude that both W(H100) and G(L50) contribute approximately comparable interactions to the weak but detectable binding of 4E10 to viral membranes, but likely through distinct molecular mechanisms. We confirmed that neither mutation affected binding to epitope peptides or gp140 constructs, consistent with the

absence of any direct role for these positions in epitope binding in the crystal structures and constraining any observed effects on neutralization to altered interactions with components of the virus outside of the defined, linear peptide epitope. In neutralization assays, the W(H100)A mutation reduced (but did not eliminate) the potency of 4E10 against a panel of four isolates, where the G(L50)E mutation increased overall potency in Fv constructs, although less than intact IgGs. Since both comparably affected membrane interactions, the combination of these two results formally separates simple, non-specific membrane binding from neutralization, showing that 4E10 can exert its broad neutralizing activity even when its independent lipid binding potential is reduced by the G(L50)E mutation. Although the W(H100)A mutation comparably reduces lipid binding, the effect of this mutation on neutralization potency is likely through a mechanism different from non-specific membrane binding, arguing against the "encounter model" of 4E10 neutralization (1). While our data do not completely exclude the encounter model, the role of W100A revealed here suggests, at the minimum, that HCDR3 performs an additional specified function in neutralizing HIV.

The more unexpected result of these studies was that the G(L50)E mutation increased neutralization potency, particularly in the context of the Fv constructs. That the effect of this mutation was less in an intact antibody may be due to the bivalent avidity swamping a minor effect, or that the potency of wild-type IgG is simply higher to begin with, minimizing the significance of the contribution. If the G(L50)E mutation does disrupt interactions with charged phospholipid head groups, the increased potency suggests that these interactions may interfere with neutralization mechanisms, although independently from peptide withdrawal, perhaps simply through sequestration in nonproductive membrane/antibody complexes. However, the contribution of these effects was not sufficient to overcome the loss of W(H100)'s contribution to neutralization through peptide extraction, and determination of the exact molecular mechanism of the G(L50)E mutation's effect on neutralization will require additional experiments.

If these residues do not contribute an important and physiologically relevant independent membrane binding mechanism, perhaps as part of the approach or encounter phase to overall binding (Fig. 11A), then why is a tryptophan residue at the tip of HCDR3 important for the neutralizing activity of 4E10? The EPR data suggest an alternate mechanism: the tryptophan residue acts as a wedge, helping to pull the MPER out of the membrane concurrent with 4E10-MPER binding or immediately following binding. This model is consistent with the known membrane-interacting behavior of tryptophan and other aromatic side chains. Multiple studies have shown that such side chains interact preferentially with the head group layer of lipid bilayers and not the acyl layer (12, 41). The classic example may be the bacterial porins, barrel-shaped integral membrane proteins that have rings of aromatic side chains on the protein surface at the lipid-solvent boundary, where they are believed to stabilize the orientation of the porin in the membrane (31, 36). Based on these results, the model we propose (Fig. 11B) is that 4E10 primarily interacts with its peptide epitope on the HIV Env protein. However, due to the partial immersion of the Env MPER in the membrane (39), the optimal interaction between MPER and 4E10 would require

FIG. 11. 4E10 interaction models, illustrating steps in hypothetical models of the interaction of 4E10 with virions. (A) In an encounter model, 4E10 first interacts with viral membrane components (1), which then direct a two-dimensional search for the epitope in the MPER (2), avoiding steric occlusion (red cross and gray arrow). (B) In a wedging model, the equilibrium orientation of MPER in the viral membrane in the absence of 4E10 (1) does not permit optimal binding of 4E10 to both protein and membrane components (2); pulling the MPER partially out of the membrane allows optimal interactions, facilitated by the side chain of W(H100) interacting with lipid head groups (3). Lipid head groups are differentially colored to indicate that the positively charged pocket of 4E10 may not interact uniformly with all phospholipids.

submersing the side chain of tryptophan H100 into the acyl layer. This unfavorable interaction is either avoided or relieved by wedging a section of the MPER out of the viral membrane, allowing optimal interactions between 4E10 and both the peptide and the membrane. Our data do not resolve whether partial lifting of the MPER occurs prior to, concurrent with, or subsequent to 4E10 binding. This model incorporates results that extend the details of the interactions between 4E10 and membrane-associated MPER peptides discussed by Sun et al. (39). It is the resulting partial extraction of MPER from the viral membrane that appears to contribute to neutralization, possibly through an induced conformational change, either locally or globally. Weak interactions directly between 4E10 and membrane components independent of interactions with the protein epitope, either nonspecifically with head group components or specifically with phosphate groups, are therefore unlikely to contribute meaningfully to either binding or neutralization. However, interactions with the viral membrane directly in conjunction with protein epitope binding may be essential for the neutralization potency of 4E10-like antibodies, which may pose the largest challenge in the design and presentation of candidate vaccine immunogens targeting MPER sequences.

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