Neutralizing Epitopes in the Membrane-Proximal External Region of HIV-1 gp41 Are Influenced by the Transmembrane Domain and the Plasma Membrane


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Failure to elicit broadly neutralizing (bNt) antibodies (Abs) against the membrane-proximal external region of HIV-1 gp41 (MPER) reflects the difficulty of mimicking its neutralization-competent structure (NCS). Here, we analyzed MPER antigenicity in the context of the plasma membrane and identified a role for the gp41 transmembrane domain (TM) in exposing the epitopes of three bNt monoclonal Abs (MAbs) (2F5, 4E10, and Z13e1). We transiently expressed DNA constructs encoding gp41 ectodomain fragments fused to either the TM of the platelet-derived growth factor receptor (PDGFR) or the gp41 TM and cytoplasmic tail domain (CT). Constructs encoding the MPER tethered to the gp41 TM followed by a 27-residue CT fragment (MPER-TM1) produced optimal MAbs binding. Critical binding residues for the three Nt MAbs were identified using a panel of 24 MPER-TM1 mutants bearing single amino acid substitutions in the MPER; many were previously shown to affect MAb-mediated viral neutralization. Moreover, non-Nt mutants of MAbs 2F5 and 4E10 exhibited a reduction in binding to MPER-TM1 and yet maintained binding to synthetic MPER peptides, indicating that MPER-TM1 better approximates the MPER NCS than peptides. Replacement of the gp41 TM and CT of MPER-TM1 with the PDGFR TM reduced binding by MAb 4E10, but not 2F5, indicating that the gp41 TM plays a pivotal role in orienting the 4E10 epitope, and more globally, in affecting MPER exposure.

Despite 3 decades of research, an effective human immunodeficiency virus type 1 (HIV-1) vaccine remains an elusive achievement. The best immunogens developed so far have elicited high-titer, HIV-1-specific Abs (Abs) that target the envelope glycoprotein (Env) on the viral membrane but have poor neutralizing (Nt) efficiency across diverse HIV-1 strains (47, 57). The membrane-proximal external region of HIV-1 gp41 (MPER) is one of the most highly conserved sequences of Env. Roughly half of the residues within this region are hydrophobic, and the MPER plays a crucial role in the fusion of the viral and cellular membranes (43). In addition, Nt epitopes within the MPER are targeted by two well-characterized broadly Nt (bNt) monoclonal Abs (MAbs) (4E10 and 2F5) (4, 15, 32, 67) and two MAbs (Z13e1 and m66.6) that neutralize a range of viral isolates but are not bNt, making this region a promising target for vaccine efforts (39, 64).

As shown in Fig. 1A, the 2F5 epitope comprises MPER amino acids (aa) 662 to 667 (ELDKWA) (36, 42), and that of 4E10 localizes to aa 671 to 676 [NWF(D/N)IT] (53, 67). 2F5 has greater potency, whereas 4E10 is effective against a greater diversity of HIV-1 isolates (4). Z13e1 limited neutralization breadth, and its epitope (aa 668 to 676 [SLWNWFDTIN]) (Fig. 1A) overlaps that of 4E10 (39). While the epitope for m66.6 overlaps substantially with that of 2F5, this MAb is less potent and broad, neutralizing only a subset of the 2F5-sensitive viruses (64). The heavy-chain variable domains of all four Nt Abs are highly mutated and possess an unusually long third complementarity-determining region of the antibody heavy chain (CDR-H3).

It has been suggested that the conserved sites on HIV-1 Env targeted by bNt Abs (such as the MPER) are “self-mimics”; thus, to elicit bNt Abs, one must break tolerance (37). In this scenario, bNt Abs are rarely produced because their self-reactive precursors are deleted by tolerance mechanisms during B-cell maturation. Support for this hypothesis initially came from studies showing that the bNt MAbs, particularly 2F5 and 4E10, cross-react with “self-antigens” consisting of cell membrane components (17). Since then, the issue of whether MAbs 2F5 and 4E10 are autoreactive has been controversial (1–3, 7, 30, 31, 45, 58). It is accepted that recognition of the
viral epitope for 4E10, and to a lesser extent 2F5, involves the surrounding lipid environment (3, 13, 18). Nuclear magnetic resonance (NMR) studies of synthetic MPER peptides in detergent micelles and lipid bilayers showed that the 4E10 epitope is partially buried in the lipid bilayer (46, 54) and is thus inaccessible to the Ab. To account for this, a two-step “encounter-docking” model was proposed (1, 54), whereby binding of 4E10 to lipid precedes binding to the MPER epitope and subsequent neutralization activity (2, 31, 56). 2F5 neutralization, on the other hand, may involve lipid binding after initial interaction with the MPER (20, 30). The idea of involvement of lipid in MAb-mediated neutralization is also supported by a decrease or ablation of neutralization upon substitution of hydrophobic residues in the CDR-H3s of either 2F5 (20, 41, 66) or 4E10 (44, 58); importantly, these mutations do not affect the ability of the MAbs to bind synthetic MPER peptides. Reversal of somatic mutations in the light-chain variable domain of m66.6 has the same effect (64), indicating a role for paratope flexibility in neutralization but not peptide binding. Taken together, these studies show that the viral epitopes for bNt MAbs 2F5 and 4E10, and hence the neutralization-competent structure (NCS) of the MPER, are critically dependent upon an interaction between MPER and lipid, regardless of the specific mechanism of Ab binding.

Despite great effort, the elicitation of high-titer Nt Abs against the MPER has not been successful (reviewed in reference 33; see also references 12 and 40). It has been suggested that the true Nt epitopes of the MPER are more complex than the reported linear sequences and that previous approaches may not have faithfully reproduced the NCS of the MPER (47, 57). Based upon evidence implicating the surrounding lipid bilayer and the TM in structuring the 2F5 and 4E10 epitopes (16, 42, 59), we examined the antigenicity of the MPER expressed on the surface of the plasma membrane (PM) and tethered to it via the TM of HIV-1 gp41 or platelet-derived growth factor receptor (PDGFR). MAbs 2F5, 4E10, and Z13e1 bound optimally to MPER-TM1, a construct comprising the MPER fused to the gp41 TM and an additional 27 aa of the cytoplasmic tail domain (CT). Through mutational analysis, we found that residues in MPER-TM1 required for binding by the bNt MAbs were associated with residues previously reported to affect neutralization; moreover, neutralization-defective mutants of bNt MAbs lost binding to MPER-TM1 in the vesicle assay but retained binding to synthetic MPER peptides. Molecular modeling revealed that the 4E10 epitope is constrained in an exposed position by the gp41 TM but not the PDGFR TM and that the C terminus of the MPER, previously thought to be surface exposed, may form part of the TM helix. These studies indicate the importance of designing immunogens that recapitulate the NCS of the MPER as seen on the virus, in the conception of an effective HIV-1 vaccine.
MATERIALS AND METHODS

Materials. MAbs 2F5 and 4E10 were provided by H. Katinger and R. Kunert (University of Natural Resources and Applied Life Sciences, Vienna, Austria) and MAbs Z13e1 and 17/9 by M. Zwick, R. Stanfield, and I. Wilson (The Scripps Research Institute, La Jolla, CA), and wild-type (WT) and mutant 2F5 Fabs [F100B(H)A and delta CDR-H3] and 4E10 MAbs [W(100)A, WAWA, and WDWD] were produced as described previously (20, 44). Goat anti-human Alexa Fluor 488 was purchased from Invitrogen (Burlington, Ontario, Canada) and recombinant gp41 (HIV-1_GM isolate; MPER- and TM-specific sequences of r-gp41 [LLGLDKWESLWNWFDIT NWLWYIKIFIMIVGGLVGLRIVFAVLSIV]) from ImmunoDiagnostics (Woburn, MA). Horseradish peroxidase (HRP)-conjugated protein A/G, HRP-conjugated goat anti-human Fab, and HRP-conjugated goat anti-mouse IgG were from Thermo Scientific (Rockford, IL). Synthetic 2F5 peptide (NH2-LQELLEDKWSLGGGC-CN92H6) was from NeoMPS (San Diego, CA) and synthetic (bio)4E10 peptide (NH2-SLWN WFDITNWLYISGC(biotin)-COOH) from the University of British Columbia NAPS Unit (Vancouver, Canada). Both were more than 95% pure.

PCR amplification of gp41 ectodomain fragment sequences. The pJW4303 vector was selected based on its successful use in DNA immunizations (25, 50) and features a highly efficient cytomegalovirus (CMV) promoter. The pDisplay vector (Invitrogen) encodes an N-terminal influenza virus hemagglutinin (HA) protein tag (YPDYDPYD), followed by the PDGF TM. pDisplay was engineered to carry cDNA encoding gp41 ectodomain fragments (the MPER, C-terminal heptad repeat [CHR], and N-terminal heptad repeat [NHR]; see Fig. 1) derived from HIV-1JR-CFSF (48); subsequently, ectodomain fragments fused to the PDGF TM were subcloned into pWJ4303 to produce vectors MPER-PDGFR, CHR-PDGFR, and NHR-PDGFR, each encoding an N-terminal HA tag (see below). PCR amplification (primers are listed in Table SI in the supplemental material) was performed using the following parameters: (i) 5 min at 95°C; (ii) 30 cycles of 1 min at 95°C, 1 min at 50°C, and 1 min at 72°C; and (iii) 10 min at 72°C. pWJ4303 was engineered to carry cDNA encoding gp41 ectodomain fragments plus TM and CT sequences of various lengths (TM1, TM2, and CT), all derived from the codon-optimized sequence of HIV-1JR-CFSF (GeneArt, Regensburg, Germany; see Fig. S1 in the supplemental material), yielding vectors MPER-TM1, MPER-TM2, MPER-CT, CHR-TM1, CHR-TM2, CHR-CT, NHR-TM1, NHR-TM2, and NHR-CT. PCR amplification (primers are listed in Table SII in the supplemental material) was performed using the following parameters: (i) 5 min at 95°C; (ii) 30 cycles of 1 min at 95°C, 1 min at 53°C, and 1 min at 72°C; and (iii) 10 min at 72°C. PCR products were subjected to double digestion with BglII-BsmI or NheI-BamHI and ligated to the pDisplay or pJW4303 plasmids, respectively.

All plasmid DNA constructs were isolated using Endo-free Midi Kits (Qiagen, Valencia, CA) from cultures of Escherichia coli DH5-α transformed by heat shock and grown overnight in LB broth containing 100 μg/ml ampicillin (LBA; Sigma-Aldrich) and verified by bidirectional DNA sequencing (Macrogen, Seoul, South Korea).

Addition of the HA tag to MPER constructs. DNA sequences encoding an N-terminal HA tag were added by PCR to the constructs encoding the MPER-TM1 Ala-substituted mutants and the WT MPER-TM1, CHR-TM1, and NHR-TM1 constructs. PCR amplification (primers are listed in Table SII in the supplemental material) was performed with reaction mixtures containing 240 to 640 ng of template DNA, 200 nM each primer, 1.5 mM MgCl2, 200 μM each deoxynucleoside triphosphate (dNTP) (Fermentas, Burlington, Ontario, Canada), and 1 U of Taq polymerase (Invitrogen) by the use of the following parameters: (i) 3 min at 95°C; (ii) 30 cycles of 1 min at 95°C, 30 s at 65°C, and 25 s at 72°C; and (iii) 10 min at 72°C. NheI-BamHI-digested PCR products were gel purified and ligated into pWJ4303 as described above.

Construction of the MPER-PS-TM1 construct. A 20-aa “spacer” comprising the first 20 residues of the sequence encoding the PDGF TM (see Fig. S1C in the supplemental material) was inserted between residues K683 and I684 of the MPER-TM1 construct. PCR amplification was performed with reaction mixtures containing 100 ng of template DNA, 25 mM each dNTP (Fermentas), 200 nM each primer (primers are listed in Table SV in the supplemental material), 2 mM MgCl2, and served as a primer in the following steps performed with a QuikChange II mutagenesis kit (Stratagene, La Jolla, CA), according to the manufacturer’s instructions. Briefly, the reaction was performed using a 50-μl final volume containing 2.5 U of Pfu polymerase, 50 ng of template DNA (pMPER-TM1), 300 ng of primer, and 1 μl of dNTP mix. The cycling parameters were as follows: (i) 30 s at 95°C; (ii) 5 cycles of 30 s at 95°C, 1 min at 52°C, and 5 min 30 s at 68°C; and (iii) 13 cycles of 30 s at 95°C, 1 min at 55°C, and 5 min 30 s at 68°C.

Site-directed mutagenesis. Ala substitutions were introduced to aa 662 to 685 of MPER-TM1 by the use of a QuikChange site-directed mutagenesis kit (Stratagene) following the manufacturer’s instructions. Where Ala residues occur in the WT MPER sequence, Gln substitutions were made instead. Briefly, reactions were performed in a 50-μl final volume containing 2.5 U of Pfu polymerase, 50 ng of template DNA (pMPER-TM1), 10 pmol each of sense and antisense primers (listed in Table SIV in the supplemental material), and 1 μl of dNTP mix. Site-directed mutagenesis cycling parameters were as follows: (i) 30 s at 95°C and (ii) 16 cycles of 30 s at 95°C, 1 min at 55°C, and 5.5 min at 68°C. A 1-μl volume of DpnI-digested reaction mixture was used to transform XL1-Blue E. coli supercompetent cells. Positive transformants were identified by blue/white screening on LBA agar and grown overnight in 2 ml of LBA broth. Plasmid DNA was purified using a GenElute Plasmid Miniprep kit (Sigma-Aldrich), and mutagenesis was confirmed by bidirectional sequencing (Macrogen; data not shown).

Protein expression in mammalian cells. The COS-7 primate kidney cell line was cultured at 37°C and 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% (vol/vol) fetal calf serum and 1 mM l-glutamine. Cells were transiently transfected with DNA constructs using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Briefly, cells were transfected with 4 μg of plasmid DNA at a ratio of 1:2.5 (microgram of DNA/microliter of Lipofectamine 2000). After 48 h, cells were lysed and analyzed for protein expression.

Cell lysate preparation. Transiently transfected cells were washed three times with phosphate-buffered saline (PBS; Lonza, Basel, Switzerland) and recovered from the plate using 1 mM EDTA (Lonza), followed by centrifugation for 5 min at 385 × g. Pellets were resuspended in 500 μl of lysis buffer (0.5 mM EDTA, 250 mM sucrose) supplemented with a protease inhibitor cocktail diluted 1:12 (Complete EDTA-free; Roche, Basel, Switzerland) and then mechanically disrupted by passage through a 22-gauge needle (30 times) and sonication (2 × 15-s pulse) with a Vircsonic sonicator (VirTis, Gardiner, NY). Cell lysates were stored at −80°C.

Confocal and fluorescence imaging. Transiently transfected COS-7 cells were seeded onto microscope cover slides (Fisher Scientific, Waltham, MA) set in the wells of a 24-well plate at a density of 2 × 105 cells/well. At 24 h later, medium was removed and slides were washed 3 times with PBS. Cells were fixed with 3.5% (wt/vol) paraformaldehyde (Sigma-Aldrich) for 20 min at 37°C. After several washes with PBS, the slides were incubated for 1 h at room temperature (RT) with MAb-diluted in PBS containing 5% (wt/vol) nonfat dried milk (NFDM; Bio-Rad, Hercules, CA). Alexa Fluor 488 goat anti-human IgG (Molecular Probes, Eugene, OR) was used as a secondary Ab for detection. Slides were mounted using Prolong with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen). Images were collected using an LSM 410 confocal laser-scanning microscope with a 63×/1.4 numerical aperture (NA) oil immersion objective (Zeiss, Oberkochen, Germany) and an IX81 motorized...
neutralized microsorce (Olympus, Tokyo, Japan). All images were analyzed using ImageJ confocal software.

**Enzyme-linked immunosorbent assays (ELISAs).** Wells of high-binding-capacity microtiter plates (Corning Inc., Corning, NY) were coated overnight at 4°C with 15 to 25 µg of total cell lysates in 35 µl of Tris-buffered saline (TBS) or with whole cells. Recombinant HIV-1NL4-3 gp41 (50 ng/well) and synthetic 2F5 (bio)4E10 peptides (200 ng/well) were used as positive controls; ovalbumin (OVA; Sigma-Aldrich) (1 µg/ well), 2% (wt/vol) bovine serum albumin (BSA; Sigma-Aldrich), and 5% (wt/vol) NFDM were used as controls for nonspecific, polyreactive binding. Wells were blocked for 1 h at RT with TBS containing 2% BSA and then washed 6 times with TBS containing 0.1% (vol/vol) Tween 20 (TBS-T) and incubated for 2 h at RT with 10 nM MAb (IgG 2F5, 4E10, Z13e1, or 17/9) diluted in TBS-T containing 1% BSA. Binding assays with mutant 2F5 and 4E10 Abs were diluted 80 nM WT and F100B/H/A 2F5 Fabs and 100 nM 2F5 delta CDR H3 Fab (20) or 10 nM WT 4E10 IgG and 4E10 IgG mutants [W100A, W100(B)A, WAWA, and WDWD] (44). After six washes with TBS-T, bound Fab was detected with goat anti-human (Fab)-HRP (1:1,500 dilution), human IgGs with protein A/G-HRP (1:2,000 dilution), and 17/9 IgG with goat anti-mouse IgG-HRP (1:1,000 dilution). Abs were diluted in TBS-T containing 5% (wt/vol) NFDM. After 1 h incubation at RT, plates were washed 6 times in TBS-T, and bound HRP was detected using 2′,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) (400 ng/ml) in citrate-phosphate buffer containing 0.03% (vol/vol) H2O2. Absorbance was measured using a Versamax microplate reader ( Molecular Devices, Sunnyvale, CA) and is reported as the optical density at 405 to 490 nm (OD405–490).

**Molecular modeling.** MPER constructs bearing the gp41 TM and PDGFR TM were modeled using the E(z) membrane depth potential (49) and the loop modeling program LOOPY (51), with short energy minimization using the CHARMM22 force field (29). The structural starting point for the models included the NMR structures of Sun et al. (54) and helices located within the membrane bilayer determined using the E(z) potential (49). In each case, models begin at residue E662 at the beginning of the MPER and continue through the predicted TM.

The hypothesized trimeric interface was guided by conservation of HIV-1 amino acid sequences (23). Redundant sequences were removed using the program CD-HIT (26). The 3% of sequences that did not contain a GxxxG motif were discarded, as these may use a distinct binding interface, leaving 2,228 unique sequences.

**Statistical analysis.** All statistical analysis was carried out using SAS statistical software version 9.2. We used analysis of variance (ANOVA) methods to compare mean Nt Ab binding to that seen with amino acid-substituted mutants expressed on the surface of the PM. This analysis was performed using the Proc GLM procedure in SAS and a Tukey adjustment for all post hoc tests to preserve the overall type 1 error. All model assumptions concerning the residuals were satisfied. Unless otherwise noted, data are expressed as means ± standard errors of the means (SEM). Where indicated, Student’s t test was used to determine differences in measured variables between experimental and control results. Significance was reported at P < 0.01 (*) or P < 0.001 (**) .

**RESULTS**

Fragments of gp41 encompassing the MPER and TM are present on the cell surface and are recognized by MAbs 2F5 and 4E10. The gene encoding gp41, from the primary HIV-1 isolate, HIV-1JR.CSF (Fig. 1A; see also Fig. S1A in the supplemental material), was used as a template for PCR amplification; HIV-1JR.CSF was selected based upon its sensitivity to neutralization by the bNt MABS 2F5 and 4E10 (68). As shown in Fig. 1B, a series of plasmids were constructed encoding gp41 ectodomain fragments starting at (i) the NHR, (ii) the CHR, or (iii) the middle of the CHR and ending at the C terminus of the MPER; ectodomain fragments were aligned, in frame, with DNA sequences encoding the gp41 TM (including the TM of the platelet-derived growth factor receptor (PDGFR-TM) and fragments of the CT (Fig. 1B; see also Fig. S1B in the supplemental material). Three panels of constructs encoding different lengths of the gp41 CT were designed to optimize the expression and antigenicity of the MPER in the context of the PM (Fig. 1B; see also Fig. S1C in the supplemental material) as follows: plasmids labeled TM1 encode the gp41 TM and the N-terminal 27 aa of the gp41 CT; TM2 plasmids encode the gp41 TM and the N-terminal 66 aa of the CT; and CT plasmids encode the gp41 TM and full-length CT. Plasmids labeled PDGFR encode the PDGFR TMs and CTS used in the pDisplay system.

Confocal and fluorescence microscopic analyses were performed on COS-7 cells transiently transfected with the TM1 panel of DNA constructs and fixed and stained with 2F5 MAb. An MPER-specific pattern of staining was observed at the periphery of the cell boundary, indicating that the recombinant proteins produced by these constructs are localized to the cell membrane (Fig. 2). In contrast, confocal microscopic analyses (see Fig. S2 in the supplemental material) and immunofluorescence analyses (see Fig. S3 in the supplemental material) with MABS 2F5 and 4E10, respectively, revealed that the MPER expressed by the CT panel of constructs localized to the cytosol of COS-7 cells. Cells expressing full-length CT fused to the NHR, CHR, and MPER ectodomain fragments had enlarged nuclei and a higher death rate, indicating that the CT in this context is toxic to cells (data not shown). Western blot analyses of cellular lysates of MPER-, CHR-, and NHR-TM1 constructs expressed in COS-7 cells, and probed with MABS 2F5 and 4E10, indicated proper expression of recombinant proteins (data not shown).

The antigenicity of the gp41 ectodomain fragments fused to either TM1 or TM2 was tested in a cell lysate ELISA. MABS 2F5 and 4E10 MABS had roughly equivalent reactivities on a given gp41 fragment (Fig. 3). However, 2F5 and 4E10 MAb reactivity with the TM2 panel was low compared to the TM1 panel. Similar results were obtained using a whole-cell ELISA method of detection, in which whole cells were grown in microwells, fixed, and probed with MPER-specific MABS (data not shown). Thus, the MPER in the context of the PM was exposed for Ab binding by all three lengths of gp41 ectodomain (MPER, CHR, and NHR). The MPER-TM1 construct was chosen for further study, as it produced optimal binding by MABS 2F5 and 4E10.

**Critical residues for Nt MAb binding to the MPER in the context of the PM mirror those required for neutralization by Nt MABS.** A series of amino acid substitutions within the MPER (aa 662 to 683) and the first 2 aa of the canonical gp41 TM (aa 684 and 685) were produced in the MPER-TM1 construct to identify residues involved in binding of the Nt MABS to the MPER presented in the context of the PM. Each mutant construct typically bore a single Ala substitution; Gly substitutions were made where Ala occurs in the WT MPER sequence (aa 667). The constructs were expressed in COS-7 cells and then tested in the cell lysate ELISA for binding by the HIV-1 Nt MABS 2F5, Z13e1, and 4E10 and by 17/9, a murine MAb that binds to a seven-residue hemagglutinin A (HA) tag (YDVPDYA) located at the N terminus of each construct (Fig. 4A to D). ELISA signals were normalized to the levels of HA expression to account for differences in the expression of each construct, and binding of Nt MABS is expressed as a percentage of binding to the WT MPER-TM1 construct. MAb binding was observed for the positive controls, including recombinant gp41 and a synthetic peptide bearing the HA epitope for 17/9.
MAb, but not for lysates transfected with “empty” vector (pJW4303), COS-7 cells, or nonspecific controls (OVA, NFDM, and BSA; see Fig. S4 in the supplemental material).

Comparison of 2F5 binding results seen within the panel of mutants indicated that 2F5 was very sensitive to replacement of Asp664, Lys665, and Trp666 with Ala (P = 0.01) compared to WT MPER-TM1; this decrease was not related to their cell surface expression, as shown by 17/9 MAb binding (Fig. 4D). However, a one-way ANOVA post hoc test showed that this association did not reach statistical significance (Fig. 4E). Together, these data indicate that the activity of the Nt MAbs in binding the MPER in the context of the PM resembles their Nt activity more than their peptide reactivities.

Antigenicity of the MPER in the context of the PM correlates with Nt activity of mutant 2F5 and 4E10 Abs bearing altered CDR-H3s. Zwick et al. initially demonstrated that Ala substitution of a Phe residue at the apex of the CDR-H3 loop of the 2F5 MAb had a profound effect upon its Nt activity (66). More recently, Julien et al. showed that whereas this same substitution decreases neutralization, deletion of the CDR-H3 apex abolishes it completely; yet both 2F5 mutants retain the ability to bind peptides bearing the 2F5 epitope in solution (20). Others have noted similar observations with mutations introduced into the CDR-H3 of 4E10 (44). Several groups have suggested that viral recognition and neutralization require the interaction of the CDR-H3s of 2F5 and 4E10 with the viral membrane (1, 2, 10, 31, 42); we further speculate that these activities also rely on the flexibility of the paratope of the MAbs, which is in part contributed to by their long CDR-H3s (see Discussion).

The requirement for an intact WT CDR-H3 in the binding of MAbs 2F5 and 4E10 to the MPER-TM1 construct in the context of the PM was analyzed. Two mutants of the 2F5 Fab were analyzed: (i) the delta CDR-H3 mutant, engineered by replacing the CDR-H3 sequence, 100TLFGVPI100F, with a Ser-Gly dipeptide, and (ii) the F100B(H)A mutant, which bears an Ala substitution of the apical Phe at position 100B (20). Four mutants bearing substitutions in one Trp or two Trps at positions W100 and/or W100(B) of the 4E10 IgG1 CDR-H3 were also assayed: the single

Of interest, binding by all three Nt Abs was increased when hydrophobic residues at or near the reported N terminus of the TM (Trp678, Ile682, and Ile684) (Fig. 4A to C) were replaced with Ala; this decrease was not related to their cell surface expression, as shown by 17/9 MAb binding (Fig. 4D). However, a one-way ANOVA post hoc test showed that this association did not reach statistical significance (Fig. 4E). Together, these data indicate that the activity of the Nt MAbs in binding the MPER in the context of the PM resembles their Nt activity more than their peptide reactivities.

**FIG 2** MPER-TM1 constructs are expressed on the cell surface and are recognized by MAb 2F5. (A to D) Confocal microscopy of COS-7 cells transiently transfected with DNA constructs encoding (A) MPER-TM1; (B) CHR-TM1; (C) NHR-TM1; and (D) cells transfected with “empty” pJW4303 plasmid. Cells were probed with 10 nM 2F5 MAb and detected with Alexa Fluor 488-labeled goat anti-human IgG. (E) Fluorescence microscopy of COS-7 cells transiently transfected with the MPER-TM1 construct and probed with 5 nM 2F5 MAb. Bound Ab was visualized with Alexa-Fluor 488-labeled goat anti-human IgG (green). Cells were counterstained with DAPI to visualize nuclei (blue).

**FIG 3** The TM1 panel of gp41-encoding constructs promotes MPER antigenicity. COS-7 cells were transiently transfected with the TM1 and TM2 panels of MPER DNA constructs. Cell lysates were produced by lysis and sonication and subsequently immobilized in microtiter wells and probed with 10 nM 2F5 IgG or 4E10 IgG and then detected with protein A/G-HRP (1:2,000 dilution). Optical density (OD405– 490) values are reported. Data are representative of the results of one of two independent experiments.
FIG 4 Profiles of reactivity of mAbs 2F5, 4E10, Z13e1, and 17/9 to cell lysates bearing WT or amino acid-substituted MPER-TM1 constructs. Constructs containing indicated single amino acid substitutions were expressed in COS-7 cells. Cells were lysed, and lysates were produced by sonication and immobilized in microtiter wells. These were probed with 10 nM Nt mAbs (2F5, 4E10, or Z13e1) and 10 nM mAb 17/9, which binds the HA tag (YPYDVPDYA) at the N terminus of each MPER construct (Fig. 1B). Bound Nt mAbs were detected with protein A/G-HRP (1:2,000 dilution) and 17/9 mAb with goat (anti-mouse IgG)-HRP (1:1,000 dilution). Abs were diluted in TBS containing 5% NFDM and 0.1% Tween 20. Binding of Nt mAbs, normalized to HA expression in order to account for differences in cell surface expression, is expressed as a percentage of binding to the WT MPER-TM1 construct (black bar; dashed line). Percent binding of constructs is reported as a ratio of the percentage of binding of normalized Nt mAb to the amino acid-substituted MPER-TM1 construct to the percentage of binding of normalized Nt mAb to the WT MPER-TM1 construct. Absorbance at 405 nm to 490 nm was recorded. Data are representative of the results of one of three independent experiments. Error bars indicate SEM. (E) Box-and-whisker plots represent the median distribution of Ab binding (2F5, 4E10, Z13e1) relative to WT MPER-TM1 expression (dashed line) to constructs bearing single amino acid substitutions. Boxes show the upper and lower quartiles with horizontal dashed lines indicative of the median. Whiskers extend from the smallest to largest values.
Ala-substituted mutants, W(100)A and W(100B)A, and the double Ala- or Asp-substituted mutants, WAWA and WDWD, respectively (44). Binding to the WT MPER-TM1 in the cell lysate ELISA was significantly diminished for the 2F5 CDR-H3 mutant Fab, F100B(H)A (<50%; P < 0.001) (Fig. 5A), compared to the WT 2F5 Fab, and binding by the delta CDR-H3 Fab mutant was almost completely absent (<6%; P < 0.001), correlating with the diminished neutralization activity of these mutant Abs (20). Binding of either mutant to the control antigens, recombinant gp41 and a peptide bearing the 2F5 epitope sequence, was not as strongly affected. The relative decrease observed across the three antigens was statistically significant for the F100B(H)A and 2F5 delta CDR-H3 Fab (P < 0.05; least-square means difference, Tukey test). Similarly, binding by the 4E10 single-substitution mutant IgG1 W(100B)A was significantly decreased (<70%; P < 0.01) (Fig. 5B) in comparison to its WT 4E10 counterpart, and binding by the double mutant, WAWA, was further decreased (<45%; P < 0.001) whereas that of the WDWD mutant was almost completely ablated (<8%; P < 0.001). This effect again mirrored the decrease observed in viral neutralization that the mutations impose (44); however, statistically significant differences were not observed for the four 4E10 mutants across the MPER-TM1, r-gp41, and 4E10 peptide antigens.

The mutant Abs were also tested for their ability to bind the panel of MPER-TM1 mutants bearing single amino acid substitutions to determine whether their mechanism of binding was similar to that of their WT counterparts. As shown in Fig. S5A in the supplemental material, Ab binding was expressed as a percentage of the 17/9 signal such that relative binding strengths could be compared among the mutant and WT Abs. Binding of WT 2F5 Fab was sensitive to substitutions in MPER-TM1 residues Glu662, Leu663, Ala667, and Leu669, as well as the core epitope, Asp-Lys-Trp, compared to the 2F5 IgG1 MAb (Fig. 4A), perhaps due to differences in Ab valency. Nevertheless, the binding profile of the F100B(H)A 2F5 Fab to the panel of MPER-TM1 mutants was similar to that of the WT 2F5 Fab, albeit being weaker overall, indicating a similar mechanism of binding to the WT Fab. As expected, the delta CDR-H3 Fab bound so weakly that a trend could not be determined.

Similarly, reactivity profiles of the 4E10 mutant IgGs on the panel of MPER-TM1 mutants were similar to that of WT 4E10, but on average their signals were lower than those of the WT IgG (see Fig. S5B in the supplemental material), and binding strength mirrored the reported Nt activity of the Ab (44). The WDWD mutant IgG bound so weakly that it was not tested. As with the data in Fig. 4B, 4E10 IgG1 reactivity was most sensitive to substitutions within the core epitope, Trp-Phe. Thus, the overall behavior of the MPER-TM1 construct and its mutants in the context of the PM correlates well with the neutralization activities of the 2F5 and 4E10 bNt Abs compared to their CDR-H3 mutant counterparts.

Replacement of the gp41 TM with that of the PDGFR reduces binding to the MPER by 4E10, but not 2F5. In order to determine the contribution of the HIV-1 gp41 TM to the presentation of the bNt Ab epitopes within the MPER in the context of the PM, the TM of HIV-1 gp41 was replaced with that of PDGFR (Fig. 1B). Confocal microscopic analyses of COS-7 cells transfected with the PDGFR-TM constructs resulted in a staining pattern and localization with 2F5 MAb (see Fig. S6 in the supplemental material) similar to those of cells expressing the HIV-1 gp41 TM (Fig. 2A to D). In the cell lysate ELISA, binding of MAb 4E10 was significantly reduced in constructs bearing the PDGFR-TM in comparison to those bearing the MPER-TM1 (P < 0.001) (Fig. 6A), whereas binding by MAb 2F5 was roughly identical between the two types of TM. Moreover, the binding strength of the three bNt MAb s normalized to 17/9 binding followed the following pattern: MPER > CHR > NHR for the TM1 constructs, whereas this pattern was followed by the 2F5 and Z13 MAbs, but not the 4E10 MAb, in binding the PDGFR-TM constructs (Fig. 6B). These results indicate that the gp41 TM influences the exposure of the 4E10 epitope within the MPER in the context of the PM.

Models of the MPER-TM1 and MPER-PDGFR explain differential binding by 4E10. Molecular models of the MPER fused to the PDGFR TM and to the HIV-1 gp41 TM were developed. Using an empirical residue-based parameter, the E(z) potential, to predict TM location and insertion in lipid bilayers (5, 49), the predicted TM segment of the PDGFR-TM fragment was separated from the MPER by an external linker of ~20 residues (see Fig. 5C in the supplemental material). This placement was confirmed by the independent membrane topology prediction program Pho-
We assumed that the external linker does not affect the MPER and thus that its structure is likely to be similar to that observed for synthetic MPER peptides associated with detergent micelles (46, 54). To test this model, a fragment comprising the N-terminal 20-aa linker of the PDGFR TM was inserted between the MPER and TM1 domains (MPER-PS-TM1) (Fig. 1B). As with the results obtained with PDGFR-TM constructs, 4E10 binding was reduced \( (P < 0.001) \) in cells expressing the MPER-PS-TM1 construct (Fig. 6A). As shown in Fig. 7A, the predicted PDGFR TM segment was oriented in the lipid bilayer using the E(z) potential and then connected to the NMR structure of the MPER produced by Sun et al. (54) via a flexible linker (Fig. 7A); this resulted in the burial of critical binding residues of the 4E10 epitope in the lipid bilayer (note the blue spheres in Fig. 7A). Strikingly, as shown in Fig. 7B and 7D for the gp41 TM, the E(z) potential predicts the TM to comprise residues 676 to 699 rather than the traditional classification of the TM comprising residues 684 to 705. The prediction that the TM includes part of the traditional MPER was further supported by Phobius, with residues 678 to 705 predicted to be TM. In support of this prediction, Yue et al. found that a gp41 construct truncated at Ala700 anchors gp41 to the membrane and mediates cell-cell fusion (61). In contrast, the original TM definition would predict that polar residues such as Asn706 and Arg707 would anchor the TM within the bilayer.

Models of gp41-TM in monomeric and trimeric conformations were also constructed. Cross-linking experiments performed on fragments of recombinant gp41 in the context of liposomes and detergent micelles (24) indicate that the TM forms trimers. Yet cryo-electron microscope analyses of whole virions conflict in this regard, with one supporting a trimeric structure (27) and the other a tripod structure (62, 63). In the monomeric model, the predicted TM was placed in the membrane by the use of the E(z) potential and then connected to the N-terminal surface.

FIG 6 Reactivity of MAb 4E10, but not MAb 2F5, is diminished in cell lysates bearing gp41 ectodomains fused to the PDGFR TM. (A) Plasmids encoding the MPER fused to one of three alternative TMs (TM1, PS-TM1, or PDGFR-TM) were used to prepare samples for the cell lysate ELISA; these were probed with 10 nM MAb 2F5, 4E10, or 17/9. (B) Plasmids encoding gp41 ectodomains (MPER, CHR, NHR) fused to two alternative TMs (TM1, PDGFR) were used to prepare samples for the cell lysate ELISA; these were probed with 10 nM MAb 2F5, 4E10, Z13e1, or 17/9. Results are reported as percent binding of Nt MAbs to constructs, normalized to HA expression. Data are from one of three independent experiments. Error bars represent SEM. Statistical significance is shown at \( P < 0.001 \) (**).
helix of the MPER that has been observed by NMR (54). The
model places the 4E10 epitope in the lipid headgroups (Fig. 7B)
instead of pointing into the lipid bilayer (Fig. 7A), potentially
making the epitope more accessible for Ab binding. For the trim-
eric model, we hypothesized that any interface between the TMs
should include the highly conserved GxxxG motif (Gly690 and
Gly694), a known TM oligomerization sequence (34) that, when
mutated, reduces gp41 TM oligomerization in bacterial mem-
branes (22). Continuing the TM model from the GxxxG motif to
the MPER places highly conserved residues at the helical interface
(denoted by black dots in Fig. 7C), as is expected for TM interfaces
(6). The E(2) potential was again used to locate the trimer in
the membrane, and the model then was connected to MPER
N-terminal surface helices (Fig. 7D; a rotated view is found in Fig.
7E). Both monomeric and trimeric models suggest a significant
difference in 4E10 epitope accessibility between the gp41 TM and
PDGFR TM constructs consistent with a critical role for the TM in
presenting this epitope for Ab binding.

DISCUSSION
Despite multiple attempts, no immunogen has successfully elic-
ted high-titer bNt Abs against the MPER. Importantly, some suc-
cess has been recently achieved by Ye et al. (60), who produced a
chimeric immunogen comprising the influenza virus HA1 do-
main fused to the full sequence of HIV-1 gp41, including its TM
and CT. Immunization of guinea pigs with this fusion protein, in
the form of a virus-like particle (VLP) or expressed by a DNA
vaccine, elicited low-titer (1:50) Nt activity against Tier-2 viruses
and a simian immunodeficiency virus-HIV-MPER (SIV-HIV-
MPER) chimeric virus; importantly, this Nt activity was com-
pletely blocked by preincubation with a peptide bearing the MPER
sequence. These results strengthen the hypothesis that the MPER
must be tethered by the TM and presented in the context of the
PM to fully form its NCS. The work of Ye et al. builds on earlier
studies which showed the affinities of MAbs 2F5 and 4E10 for their
epitopes on a gp41 fragment, comprising the MPER and TM and
part of the CT, were increased when associated with lipid (16, 42). Their results, taken together with the results reported here, suggest that conclusions from NMR studies of synthetic MPER peptides and their interaction with lipid micelles (46, 54), which showed the residues critical for 4E10 MAb binding to be buried in a lipid micelle, can be explained by the absence of the TM (and possibly the CT) in these peptides. We offer a model based upon biochemical evidence to explain this discrepancy; the NCS of the MPER, and especially its 4E10 epitope region, depends upon both a lipid environment and the HIV-1 gp41 TM, with each contributing to its conformation.

The majority of previous work analyzing the MPER and the bNt MAbs that recognize it was performed with synthetic peptides whose sequence is limited to the MPER or recombinant, soluble proteins (reviewed in reference 33). We show here that the TM plays an important role in epitope presentation when the TM is displayed in the context of the PM (Fig. 4 and 6) and provide molecular models to explain the difference in exposure of the 4E10 epitope observed between MPER anchored by the PDGFR TM versus the gp41 TM. We speculate that the extended external region of the PDGFR-TM allows the MPER C terminus freedom to move in the PM, resulting in the burial of core residues in the 4E10 epitope (Fig. 7A); this was confirmed by the results obtained using this external region as a linker between the MPER and gp41 TM in the MPER-PS-TM1 construct (Fig. 6A). While it may have affected the structure of the MPER directly, a relatively hydrophilic, flexible linker is not expected to strongly affect the hydrophobic 4E10 epitope. Instead, it is more likely that the conformational constraints imposed by the adjacent gp41 TM directly affect exposure of the 4E10 epitope (Fig. 7). Our analysis of the gp41 TM predicts that it spans residues 676 to 699; thus, the TM begins in what is currently described as the C-terminal region of the MPER (aa 676 to 683). We suggest that this region acts to tether the MPER closely to the PM interface (Fig. 7B), and in doing so, this constraint more fully exposes the 4E10 epitope.

Further, based upon a model initially proposed in studies by Cleveland et al. (11, 52), the TM of MPER-TM1 construct could span the membrane twice; conflicting views exist regarding the degree of membrane spanning of the gp41 TM (28). The TM also appears to play a role in the overall exposure of the MPER, as selected amino-acid substitutions in this region of the TM affect binding by all three Nt MAbs (Fig. 4). Though the results did not reach statistical significance, a trend was observed when Ala substitution of hydrophobic amino acids surrounding the N terminus of the TM, Trp678, Ile682 and Ile684 increased the binding of all three Nt MAbs, whereas Ala substitution of a hydrophilic aa in that region, Lys683, decreased MAb binding. Thus, we propose that residues in the N terminus of the TM directly affect positions of the MPER’s C-terminal region. Exposure of the MPER, and the 4E10 epitope in particular, may also be affected by the quaternary structure of the TM (monomeric versus trimeric) (Fig. 7B versus 7D); this awaits further study.

The TM of HIV, like that of PIV5, is unusually long compared to other TMs, possibly to stabilize a fusion intermediate (5). For these long TMs, decreased hydrophobicity at one end can be compensated by sliding the other end of the TM further into the membrane. In this case, replacement of hydrophobic residues near the TM’s N terminus would make movement out of the membrane more favorable, thus increasing the accessibility of MPER epitopes. On the other hand, loss of a hydrophilic residue, such as Lys683, may cause deeper burial of this region, which in turn may weaken Ab binding. Others have found that binding of 4E10 and 2F5 is inversely correlated to the extent of immersion of epitope residues (14), supporting the role of membrane anchoring in exposing the MPER. The recent observation of 4E10-resistant strains that have substituted hydrophilic and aromatic residues in this region (38) also supports a role for membrane anchoring in 4E10 binding. However, the full effect of increased exposure of epitopes in the MPER on immunogenicity has yet to be evaluated.

Our results also suggest that the presence of gp41 domains N-terminal to the MPER does not add to bNt MAb binding, as it was not enhanced for constructs comprising the CHR and NHR domains in addition to the MPER (Fig. 6B). One caveat regarding our experiments is that they involved recombinant, transiently expressed MPER constructs on the surface of a primate kidney cell line instead of the native viral membrane. Structural differences may exist between the viral and cellular forms of the MPER, as the CT has been shown to produce antigenic differences between the Env on viral particles versus the same Env on the cell surface (52). For native gp41, which exists in numerous prefusogenic and fusogenic states, kinetic restrictions may limit bNt Ab binding to the MPER, whereas the recombinant constructs could allow better exposure of the MPER to bNt Ab binding; however, better exposure may not translate into better immunogens if obstruction by the domains upstream from the MPER must be overcome by a Nt Ab. Nevertheless, many of the amino acid substitutions we found to affect Ab binding have also been shown to affect neutralization (39, 65), as is consistent with these constructs acting as functional mimics of the NCS. Our results also agree with those of Maeso et al. (30) who showed differential binding between 2F5 Fab and the Fab mutants of Julian et al. (20) to MPER peptides in the context of the lipid bilayer; the differences in binding mirrored their Nt activity.

We envision the NCS of the MPER to be structurally akin to a “speed bump” on the surface of the viral membrane that is constrained by a TM tether; as our results suggest, a highly flexible Ab paratope is required for binding to this structure and subsequent viral neutralization. Crystal structures of 2F5 (9, 19, 42) and 4E10 (10) Fabs bound to synthetic MPER peptides show that the CDR-H3 apex of these Fabs extends well beyond the peptide, and yet amino acid substitutions or deletions at the CDR-H3 apex of 2F5 (20, 66) or 4E10 (2, 44, 58) decrease or ablate neutralization, respectively. While binding to peptides or gp41 in solution is not affected by alterations in the CDR-H3 apex, binding to the MPER in our cell lysate ELISA was reduced by half for the 2F5 Fab mutant, F100B(H)A, and completely ablated for the 2F5 Fab mutant in which the CDR H3 loop was truncated; these losses in MPER reactivity correspond to the partial and full loss of Nt activity for these Fabs, respectively, compared to WT 2F5 Fab, and similar observations were found for 4E10 and its mutant Abs. Yet these mutant Abs still retained the ability to bind to peptides in solution and, in our study, to peptides bearing their respective epitopes and recombinant-gp41 adsorbed to polystyrene. Thus, it appears that MPER-TM1 antigenicity closely follows the neutralization activities of the bNt MAbs and their mutants. These results provide further support for the continued development of the MPER-TM1 construct as an immunogen.

Notably, this same effect, albeit present to a lesser degree, has been shown for peptides that are directly adsorbed to ELISA wells both here and by others (20, 66). Taken together, the results suggest a mechanism in which a long CDR-H3 loop enhances
paratope flexibility, allowing it to open up to engage the speed bump conformation of the MPER in the context of the PM; this very likely also involves direct interaction of the CDR-H3 with the PM. Interestingly, in separate work, we obtained similar results with two other HIV Nt MAbs, m66 and m66.6, which bind an epitope that mostly overlaps with the 2F5 epitope (64). The m66 and m66.6 Abs are identical, except m66 has 6 fewer amino acid replacements than m66.6 and the neutralization breadth of m66 is more limited than that of m66.6, and yet they bind MPER synthetic peptide with similar affinities. In similarity to the other mutant Abs with lower Nt activity, the m66 Fab bound the MPER-TM1 more weakly than the m66.6 Fab, and yet both Fabs were affected by the same amino-acid substitutions in the MPER-TM1, indicating that their overall binding preferences were identical. Thus, in this case, a difference in Nt activity, caused by differences in the variable domain of the immunoglobulin light chain (VL), not the heavy chain ( VH) or the CDR-H3, mirrored binding to the MPER-TM1 and is consistent with our hypothesis that Ab paratope flexibility is required in binding the NCS of the MPER. Future work with a recently discovered MPER-specific Nt MAb, CAP206-CH12, possessing overlapping epitopes with the 4E10 and Z13e1 MAbs, sharing the same germ line heavy and variable kappa light chains as the 4E10 MAb, and exhibiting CDR-H3 sequence similarities to Z13e1 MAb (35), should further clarify the role of Ab paratope flexibility in neutralization.

As mentioned above, Ye et al. reported that guinea pigs immunized with an influenza virus HA/HIV-1 gp41 chimera protein elicited low-titer, MPER-specific Nt Abs (60). Low-titer, MPER-binding Abs that lacked Nt activity were elicited in preliminary rabbit-immunization studies using the DNA constructs described here (unpublished data); if this was not merely an issue of low-titer Ab, then we must conclude that we had not fully replicated the NCS of the MPER. However, both studies were impeded by a major hurdle that must be overcome in designing an MPER-targeting vaccine: elicitation of low-titer anti-MPER Abs. We speculate that titers are low because of low expression or copy numbers of MPER-TM-CT fragments presented in the context of the PM, for current DNA and VLP vaccines, respectively. It may be possible to produce synthetic, liposome- or nanoparticle-based vaccines that present the MPER-TM in the context of a virus-like lipid bilayer, recapitulating the viral NCS. If that can be achieved, we suggest a strategy for producing high-titer Nt Ab responses based on DNA or VLP priming immunizations followed by boosts comprising liposome or nanoparticles bearing high-copy-number MPER. The recent success of Ye et al. (60) indicates that a strategy focused on improving Nt Ab titers is promising. Coupled with this, further work in designing immunogens that more faithfully mimic the NCS of the MPER in vivo should further inform vaccine design and improve bNt Ab responses.

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