HIV Hot Paper

Deutsche Ausgabe: DOI: 10.1002/ange.201508421
Internationale Ausgabe: DOI: 10.1002/anie.201508421

Trimerization of the HIV Transmembrane Domain in Lipid Bilayers Modulates Broadly Neutralizing Antibody Binding

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Abstract: *The membrane-proximal external region (MPER) of HIV gp41 is an established target of antibodies that neutralize a broad range of HIV isolates. To evaluate the role of the transmembrane (TM) domain, synthetic MPER-derived peptides were incorporated into lipid nanoparticles using natural and designed TM domains, and antibody affinity was measured using immobilized and solution-based techniques. Peptides incorporating the native HIV TM domain exhibit significantly stronger interactions with neutralizing antibodies than peptides with a monomeric TM domain. Furthermore, a peptide with a trimeric, three-helix bundle TM domain recapitulates the binding profile of the native sequence. These studies suggest that neutralizing antibodies can bind the MPER when the TM domain is a three-helix bundle and this presentation could influence the binding of neutralizing antibodies to the virus. Lipid-bilayer presentation of viral antigens in Nanodiscs is a new platform for evaluating neutralizing antibodies.*

The membrane proximal external region (MPER) is a portion of the envelope glycoprotein gp41 on the surface of HIV virions. The MPER is critical to membrane fusion and infection, and is one of the most highly conserved regions of HIV.[2] A number of broadly neutralizing antibodies including 4E10, Z13e1, 2F5, and 10E8 have been identified that target overlapping regions of the MPER, and a broad analysis of patient sera suggest that MPER-directed antibodies are more common than previously thought[3]. Neutralizing antibodies identified from HIV+ individuals have been shown to provide protection against intravenous and mucosal challenge in passive transfer animal studies, suggesting that a neutralizing antibody response could protect against HIV.[4] Despite the apparent linear nature of described MPER epitopes, the design of effective MPER immunogens has had limited success, suggesting the MPER is more than a linear peptide epitope.[5] Thus the MPER is ripe for a reverse vaccinology approach to elucidate the interactions between known broadly neutralizing antibodies and their epitopes in order to direct the iterative design of immunogens.

The structure of the MPER is largely unknown in the context of Env trimers, despite high-resolution structural information regarding Env trimers both on the virion surface and in soluble forms. Cryo-EM data of virion surfaces show low density and low signal to noise at the MPER, suggesting less protein mass or greater disorder in this region.[6] Recent cryo-EM[1] and X-ray crystallography structures[7] of stabilized soluble gp41–gp120 trimers required removal of the MPER to obtain stable constructs. The conformation of the MPER is still an open question of great interest and import to antigen design and general understanding of HIV.

Recognizing the importance of the membrane in understanding the MPER, the MPER has been presented in association with micelles and liposomes or by anchoring MPER peptides with lipids or membrane surface-binding motifs.[3b,10] The native HIV particle, however, anchors the MPER through a transmembrane (TM) domain fused to its C-terminus. We suggest that an MPER peptide containing a TM domain in a lipid bilayer should be thought of as an

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201508421.

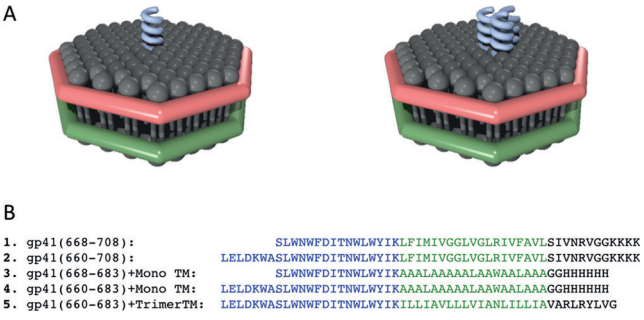

Figure 1. A) Cartoon model of monomeric and trimeric MPER-TM peptides presented in a Nanodisc. A planar lipid bilayer is stabilized and solubilized by a modified apolipoprotein described by Sligar and co-workers.[11] B) Peptides used in this study. Residues in blue correspond to the MPER, residues 661–683 of Env (HxB2 numbering). Residues in green correspond to the transmembrane domain from HIV (peptides **1** and **2**), a designed monomeric TM domain (peptides **3** and **4**), or a designed trimeric TM domain (peptide **5**).

integrated unit. In order to model the entire MPER-TM domain in the context of a defined lipid bilayer, we chose to present synthetic MPER-TM peptides in protein-stabilized, 10 nm diameter lipid Nanodiscs (Figure 1 A). Nanodiscs provide a soluble assembly of membrane proteins embedded in a native-like lipid bilayer that retains native structure and function.[11]

Five synthetic peptides were designed, representing combinations of two different MPER lengths and three different transmembrane domains (Figure 1 B). All peptides used the HxB2 gp41 sequence for the MPER and or TM domain, except for a N674D mutation to enable Z13e1 binding, and all numbering corresponds to the HxB2 reference sequence. MPER-Nanodiscs were assembled, purified, and characterized according to the methods of Sligar and co-workers.[11,12] Briefly, membrane scaffold protein, cholate-solubilized dimyristoyl phosphatidylcholine and MPER peptides were combined in aqueous buffer containing 24–40 mM sodium cholate. Self-assembly was initiated by the removal of detergent affected by BioBeads or by dialysis. Assembled Nanodiscs were purified by size-exclusion chromatography (Figure S2 in the Supporting Information), and analyzed by dynamic light scattering to ensure consistent sizing.

MPER-TM peptide **1**, gp41(668-708), contains the epitopes for both Z13e1 and 4E10 as well as the HIV TM domain while Peptide **2**, gp41(661-708), extends the MPER to include the epitope for 2F5. (Figure 1 B) These peptides, incorporated into Nanodiscs, were immobilized onto polystyrene plates and incubated with the HIV-neutralizing antibodies 2F5, Z13e1, and 4E10 to determine binding affinity via ELISA (Table 1, rows 1 and 2). No avidity effects were observed as evidenced by the similar affinity of Z13e1 IgG and F_{ab} fragment. As a negative control, peptide **1**, which does not contain the epitope for 2F5, exhibited no detectable binding to 2F5 ($EC_{50} > 2$ μM).

Since the oligomerization state of the MPER is unknown, chimeric MPER peptides incorporating a monomeric TM domain designed by Deber and co-workers[13] were incorporated into membranes and examined for binding to 4E10, Z13e1, and 2F5. The shorter peptide **3**, gp41(668-683) + MonoTM, showed strong affinity for 4E10 and Z13e1, but no affinity for 2F5 since it does not contain the epitope for 2F5 (Table 1, row 3). The longer peptide **4**, gp41(661-683) + MonoTM, however, exhibited markedly diminished affinity for 4E10 (25-fold). Similarly, affinity for 2F5 (30-fold) was an order of magnitude weaker than peptide **2**, which contains the HIV TM domain (Table 1, rows 2 and 4). Notably, peptide **4** showed no evidence of binding to Z13e1 (Table 1, row 4, column 2). The monomeric transmembrane domain clearly presents the MPER to bnAbs differently than the HIV transmembrane domain. Collectively, the full MPER sequence when fused to the monomeric TM domain appears to be a poor mimic of the conformations best recognized by the broadly neutralizing antibodies 4E10, Z13e1, 2F5, and 10E8 (Table 1, row 3) when compared to shorter MPER peptides as judged by binding affinity. These observations question the suitability of the monomeric TM domain for MPER epitope presentation.

Since the glycoprotein spikes assemble as trimers distal to the viral membrane,[14] it is possible (although not required) that their TM domains associate with the same stoichiometry. DeGrado and co-workers have described a designed transmembrane domain that incorporates into lipid bilayers as a parallel three-helix bundle.[15] To further examine the effect of the oligomerization state on MPER presentation, we designed peptide **5** (MPER-trimerTM) to display the gp41 MPER domain in the context of this stable three-helix bundle TM domain. Peptide **5** was incorporated into Nanodiscs and evaluated for binding to 4E10, Z13e1, and 2F5. Trimeric peptide **5** showed a highly similar binding profile to peptide **2**, which contains the same MPER sequence but the native HIV TM domain (Table 1). Gel electrophoresis of MPER-TM peptides supports the hypothesis that peptide **2** can exist as a trimer in environments such as SDS micelles along with a dimeric state (Figure 2).

The broadly neutralizing antibody 10E8 neutralized 98% of tested viral isolates with potency comparable to the most potent neutralizing antibodies known, further enhancing the MPER as a retrovaccinology target.[3d] 10E8 recognizes an

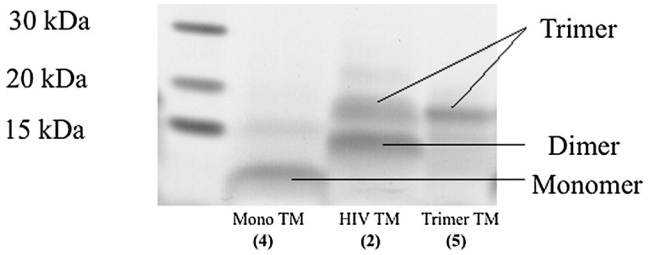

Figure 2. Peptide **2**, MW 6390 Da and containing the HIV TM domain, migrates by electrophoresis as a mixture of dimers and trimers. Peptide **4**, MW 5966 Da and containing a monomeric TM domain, migrates as a monomer, and peptide **5**, MW 6107 Da, containing a trimeric TM domain, migrates as a trimer.

Table 1: ELISA binding assays.[a]

	Peptide	4E10 IgG	Z13e1 IgG	2F5 IgG	10E8 IgG	4E10 WAWA	2F5 CDRH3 mutant	Z13e1 Fab
1	gp41(668-708)	6.0 ± 0.5	161 ± 33	> 2000	N.D.	> 2000	> 1000	109 ± 10
2	gp41(661-708)	1.8 ± 0.2	20 ± 4	2.3 ± 0.3	3.6 ± 0.4	> 2000	> 1000	17 ± 3
3	gp41(668-683) + Mono TM:	3.1 ± 0.2	13 ± 2	> 2000	N.D.	> 2000	> 1000	24 ± 2
4	gp41(661-683) + Mono TM:	74 ± 12	> 2000	26 ± 2.7	105 ± 15	> 2000	> 1000	> 2000
5	gp41(661-683) + TrimerTM:	1.5 ± 0.2	56 ± 8	0.2 ± 0.1	1.4 ± 0.2	> 2000	> 1000	19 ± 4

[a] EC_{50} values listed in nM. Note in particular columns 7 and 8, showing that none of the Nanodisc-presented MPER-TM peptides bound non-neutralizing antibodies. Peptides **1** and **3** do not contain the 2F5 epitope and thus did not bind. N.D. = not determined.

epitope broadly defined as residues 656–683, therefore, peptides **2, 4**, and **5**, containing the longer MPER sequence and presented in Nanodiscs, were examined for 10E8 affinity. Peptide **2**, containing the native HIV TM domain, bound 10E8 with high affinity $EC_{50} = 3.6 \pm 0.4$ nM, similar to peptide **5**, containing a designed trimeric TM domain. In contrast, peptide **4**, containing a monomeric TM domain, had 30-fold weaker affinity.

An additional examination of the membrane context for MPER peptides anchored by a transmembrane domain was performed by analyzing the affinity of the mutant antibodies 4E10 WAWA (mutations: W100A W100BA) and 2F5 CDRH3 (mutations: L100AS and F100BS).[10a,d] These antibodies contain mutations (Trp to Ala and L100AS and F100BS that reduce the hydrophobicity of the CDRH3 loop and were designed to weaken the interaction between the Ab and the membrane surface. These mutant antibodies retain high affinity to soluble peptides, but completely lose neutralization potency. As such, they serve to test whether the integrated MPER domains could distinguish between neutralizing and non-neutralizing binding interactions. Neither 4E10 WAWA nor its comparable 2F5 CDRH3 mutant exhibited any detectable binding to peptides **1**–**5** in Nanodiscs.

These ELISA-based binding assays used immobilized MPER-Nanodisc assemblies, which, while an established format for membrane protein presentation, could raise concerns about surface effects. To measure association constants in solution, we utilized label-free backscattering interferometry (BSI) performed on an instrument described by Baksh et al.[16,17]

BSI detects small changes in the refractive index of a solution caused by interactions between molecules, and is well suited for the study of antibody binding without tags or immobilization.[18] In all cases, the binding constants determined by BSI agreed well with those obtained from ELISA (Table 2 vs. Table 1), providing confidence that the observed interactions are not perturbed by immobilization of Nanodisc-presented peptides. Notably, Z13e1 was found to not bind to the monomeric peptide **4** incorporated into Nanodiscs (Table 2, row 2), while peptide **2**, containing the HIV TM domain, and peptide **5**, containing the trimeric TM domain showed strong binding (Table 2, rows 1 and 3 compared to Table 1, rows 1 and 5, respectively).

Table 2: Solution-phase binding constants (K_d in nM) determined by backscattering interferometry.

	Peptide	4E10 IgG	Z13e1 IgG	2F5 IgG
2	gp41(661-708)	2.3 ± 0.7	4.8 ± 1.6	0.4 ± 0.1
4	gp41(661-683) + Mono TM	1.1 ± 0.8	>400	7.1 ± 2.4
5	gp41(661-683) + TrimerTM	0.3 ± 0.2	21 ± 8	3.0 ± 1.0

This work supports a model in which the HIV TM domain can exist as a three-helix bundle that presents the MPER in the context of the phospholipid membrane surface. First, the neutralizing antibody binding profile of the HIV MPER-TM domain is remarkably similar to MPER chimeras fused with a trimeric TM (correlation p value < 0.0001), but quite distinct from monomeric chimeras (Figure S4). In the context of long MPER sequences, these monomeric TM peptides displayed reduced binding to 4E10 (10-fold) and no measured binding to Z13e1 (>100-fold). Solution measurements obtained by backscattering interferometry confirmed these results. Second, while MPER peptides with a designed monomeric TM migrate as monomers by SDS gel electrophoresis, MPER-HIV TM peptides migrate primarily as trimers, even after boiling in SDS (Figure 2). Such TM domain trimerization is also consistent with modeling studies that identified an energetically reasonable trimerization interface.[10c] Although previous studies have identified other portions of Env that direct trimerization,[6b,19] our results suggest that the isolated MPER-TM is sufficient for trimerization in a lipid bilayer.

These results show the importance of both membrane localization and TM-dependent oligomerization state of MPER epitopes - neither condition alone is sufficient. Previous reports have shown that although association with a membrane can enhance neutralizing antibody affinity to MPER peptides,[20] they often retain significant affinity to non-neutralizing MPER antibodies.[10d] Similarly, presentation of the MPER on stable trimeric gp41/gp120 constructs[21] have produced soluble trimers that show reduced or no binding to MPER-targeted neutralizing antibodies[8,22] with a preference of binding residual monomeric fragments.[23] Furthermore, fusion of the MPER to either terminus of 3-helix bundle domains abrogates binding to neutralizing antibodies.[24]

In contrast, Nanodisc-presented trimeric MPER-TM peptides bind with a native-like antigenic profile—showing strong interactions with neutralizing antibodies 10E8, 4E10, 2F5, and Z13e1 without also binding non-neutralizing mutants of these antibodies. We propose that membrane presentation via a parallel 3-helix bundle transmembrane domain is necessary to balance flexibility and structure to recapitulate neutralizing antibody binding as well as the lack of non-neutralizing antibody binding.

These results indicate that neutralizing antibodies recognize a membrane-presented trimeric version of the MPER. The MPER-TM domain is absent in the recent EM and crystal structures of stabilized SOSIP trimers. However, the overlap between the backbone trace of gp41 628–660, corresponding to the C-heptad repeat, and the 10E8-bound MPER structure allows these two structures to be considered together. Even with considerations for flexibility taken into account, the trajectory of the C-heptad repeat region suggests that the N-terminal portions of the MPER (gp41 661–683) are located ≈ 30 Å apart. In principle, the MPER domains could turn inward and connect with a three-helix bundle TM domain. However, in this conformation the MPER would be highly occluded and unlikely to engage MPER binding antibodies. Another structural interpretation that is consistent with these data is depicted in Figure 3. Instead of turning inward, the MPER could continue to propagate the observed CHR helix and then turn near Trp670. In this model, the MPER helix from residues 671–683 could lay parallel to the membrane surface. This model would require a second turn

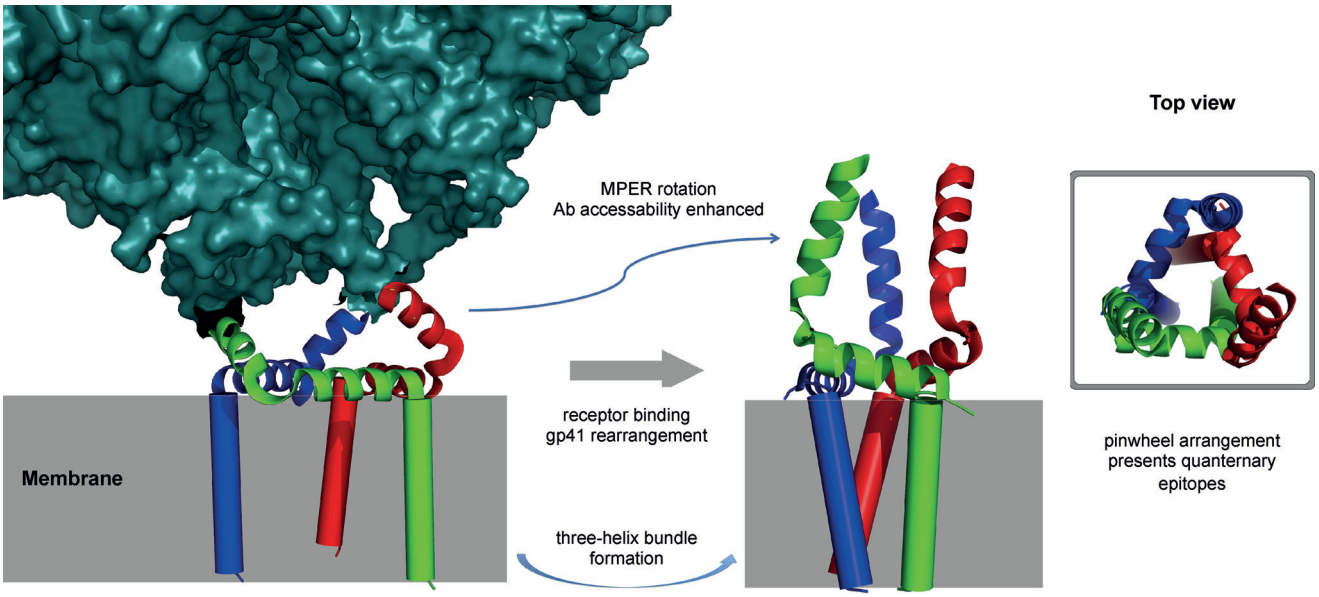

Figure 3. A structure-inspired cartoon model of gp41 illustrating a TM rearrangement that would expose MPER trimers to bn-antibodies. The space-filling model (teal) shows a soluble version of gp41 taken from Ward and co-workers 3J5M.pdb,[1] the MPER shown in ribbons is taken from the structural coordinates of the MPER bound to 10E8 (4G6F.pdb[3d]), and the cylinders represent generic helices depicting the TM domain. The left panel depicts the unliganded Env spikes tethered to the membrane via three spatially separated TM helices. The right panel depicts the MPER and TM domain following gp41 rearrangements following CD4 binding, which would better accommodate antibody binding and enable an energetically preferred association of the three TM helices. In both states the MPER is depicted in the 10E8 conformation. TM trimerization places three copies of the MPER in a pinwheel arrangement, suggesting the possibility that the MPER from individual subunits could directly interact and present discontinuous epitopes that would not be present in other Env conformations.

around Lys683 to direct the TM into the membrane, consistent with recent NMR structures of the MPER in hexafluoroisopropanol and DPC micelles.[25] Using the crystal structure of the MPER peptide bound to 10E8 as a guide,[3d] this arrangement would place the TM domains ≈30 Å apart but would make the MPER more accessible to antibodies such as 10E8.

So what is the role of the three-helix bundle? If the TM domains in unliganded Env are monomeric, conformational changes in gp41 induced upon CD4 binding could be coupled to TM trimerization—a thermodynamically favorable process that could facilitate further conformational changes necessary to form the pre-hairpin intermediate.[26] Though the exact mechanism of neutralization by MPER bnAbs is unknown, there is general agreement that these antibodies more readily interact with CD4-bound forms of Env.[27] A model for this trimeric MPER conformation is shown on the right side of Figure 3, again inspired by the 10E8-bound conformation of the MPER. At the junction of the MPER and the TM domain (three-helix bundle) the peptide turns such that residues 671–683 lie flat on the membrane surface as seen in micelle models. Around W670, the MPER peptide turns and extends away from the membrane consistent with the helices seen in the structure of 10E8. This orientation of the MPER enables antibody binding without clashes between the antibody and Env. Importantly, instead of the MPER extending radially from the center of the transmembrane trimer, our model places the three copies of the MPER in a pinwheel arrangement, suggesting the possibility of MPER-MPER contacts. These contacts are built into our trimeric MPER-TM mimics but are not possible in previous studies. In particular, this arrangement would be compatible with discontinuous quaternary epitopes existing between MPER subunits, similar to those observed with glycan-binding antibodies targeting interfaces between protein subunits.[28]

HIV Env is known for exploiting the latent oligomerization potential of peptides; in particular six-helix bundle formation from the N-heptad and C-heptad repeat helices take advantage of this general model.[26a] Releasing the latent trimerization energy of the HIV TM domain may be important to membrane fusion and cell infection, partially explaining its remarkable sequence conservation, and lending credence to a model where the HIV TM domain has a strong functional role in additional its role in spanning the viral membrane.

Typical structure-guided epitope mimicry leverages knowledge of the structure of an antigen in an antibody-recognized conformation.[29] To accommodate highly conformationally flexible peptides such as the MPER, however, this approach can be inverted: we have used neutralizing and non-neutralizing antibodies to evaluate structural mimics of the MPER. Our results show that both membrane anchoring and trimeric presentation are required to replicate a native-like antigenic profile. Consideration of an expanded, membrane anchored, and trimerically presented epitope may elucidate important structural features of the MPER and TM domain relevant to infection.

Acknowledgements

This research was supported by the California HIV/AIDS Research Grants Program, D10-SRI-30 (T.M.R.), International AIDS Vaccine Initiative (P.E.D.), and NIH EB015663 (M.G.F.), GM33775 (S.G.S.), AI114401 (M.B.Z.), and GM098871 (P.E.D.). We thank V. Mitch Luna, C. David Stout, Johannes S. Gach, Arthur S. Kim and Florence Brunel for assistance and advice.

Keywords: antibodies · HIV · membrane proteins · nanostructures · peptides

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 2688–2692
Angew. Chem. **2016**, *128*, 2738–2742

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Received: September 21, 2015
Revised: November 4, 2015
Published online: January 22, 2016

