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Deletion of fusion peptide or destabilization of fusion core of HIV gp41 enhances antigenicity and immunogenicity of 4E10 epitope

Jing Li [a,b,1], Xi Chen [a,b,c,1], Shibo Jiang [c,*], Ying-Hua Chen [a,b,*]

[a] Laboratory of Immunology, Department of Biology, Tsinghua University, Beijing 100084, PR China
[b] Beijing Key Laboratory for Protein Therapeutics, Beijing 100084, PR China
[c] Laboratory of Viral Immunology, Lindsley F. Kimball Research Institute, New York Blood Center, NY 10065, USA

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ABSTRACT

The human monoclonal antibody 4E10 against the membrane-proximal external region (MPER) of HIV-1 gp41 demonstrates broad neutralizing activity across various strains, and makes its epitope an attractive target for HIV-1 vaccine development. Although the contiguous epitope of 4E10 has been identified, attempts to re-elicit 4E10-like antibodies have failed, possibly due to the lack of proper conformation of the 4E10 epitope. Here we used pIg-tail expression system to construct a panel of eukaryotic cell-surface expression plasmids encoding the extracellular domain of gp41 with deletion of fusion peptide and/or introduction of L568P mutation that may disrupt the gp41 six-helix bundle core conformation as DNA vaccines for immunization of mice. We found that these changes resulted in significant increase of the antigenicity and immunogenicity of 4E10 epitope. This information is thus useful for rational design of vaccines targeting the HIV-1 gp41 MPER.

Design of immunogens that are capable of eliciting broadly neutralizing antibodies against human immunodeficiency virus type 1 (HIV-1) is a major goal for vaccine development. Regarding its critical roles in viral infection, the envelope glycoprotein (Env) transmembrane subunit gp41 has served as an attractive target for HIV vaccine design [1]. The gp41 is composed of several relatively conserved regions, including fusion peptide (FP), N- and C-terminal heptad repeats (NHR and CHR, respectively), membrane-proximal external region (MPER) and transmembrane domain (TM), all of which participate in virus-to-cell fusion and virus entry into the target cell [2–6].

Antibodies against HIV-1 gp41 could be classified into clusters I and II [7–9]. Cluster I antibodies react with the immunodominant region of gp41 (aa 579–613), most of which are non-neutralizing. Cluster II antibodies recognize the epitopes in MPER (aa 644–684), including several rare broadly neutralizing human monoclonal antibodies (MAbs), e.g., 2F5, 4E10, and Z13 [10–14]. However, immunization of animals with antigens containing the gp41 MPER could not induce neutralizing antibodies [15,16], which may result from the failure of free peptides to present a proper conformation mimicking that of the native epitope in the context of the virus membrane. Thus, providing an appropriate micro-environment and maintaining relevant epitope conformations may increase the possibilities of eliciting an effective neutralizing antibody response.

To this end, we investigated the potential effect of changing the structure and conformation of gp41 ectodomain presented on the cell surface on the antigenicity and immunogenicity of epitopes located in the gp41 MPER, particularly the 4E10 epitope, because it is highly conservative and 4E10 MAb exhibits neutralizing activity against a broad spectrum of HIV-1 strains [10]. We generated a panel of pIg-tail-fused eukaryotic expression plasmids encoding the HIV-1_{HXB2} gp41 ectodomain and its fragments with deletion of FP and/or introduction of L568P mutation in NHR. L568 is a critical residue involved in formation of the conserved hydrophobic pocket that is important for stability of the gp41 six-helix bundle (6-HB) core [17] and substitution of a leucine with a proline is expected to be disruptive of an α-helical coiled coil [18]. These plasmids were transfected into CHO cells to express gp41 ectodomain and its fragments for studying the antigenicity of 4E10 epitope. The plasmids were also used as DNA vaccines to immunize mice for investigating the immunogenicity of the 4E10 epitope.

Materials and methods

Reagents and cell lines. The peptide containing twice-repeated 4E10 epitope (4E10P2: CGNWFNITGNWFNIT) were synthesized by the SBS Genetech Technology Co., Shanghai, China. CHO cells

* Corresponding authors. Fax: +1 212 570 3099 (Y.-H. Chen), +86 10 6277 1613 (S. Jiang).
E-mail addresses: sjiang@nybloodcenter.org (S. Jiang), chenyh@mail.tsinghua.edu.cn (Y.-H. Chen).
[1] These authors are contributed equally to this work.

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were cultured in glutamine-deficient minimal essential medium (GMEM-S) containing 400 mM methionine sulfoximine (Sigma, St. Louis, MO). FITC-conjugated goat anti-mouse IgG was purchased from DAKO (San Jose, CA). N36(L8)C34 and NC(L568P) were expressed and purified as previously described [19].

Construction of Env glycoprotein expression plasmids. The plasmids encoding intact gp41 ectodomain, gp41ect$_{-\Delta FP}$ (deletion of FP), gp41ect-L568P (L568 mutation in NHR), gp41ect-L568P$_{-\Delta FP}$ (both L568 mutation and FP deletion), and gp41 MPER were constructed using pSK-gp160 plasmid. PCR reactions were carried out using a common reverse primer 5′-TTTGGATCCACTTACCTGTTTTTATACC ACAGCCAATTT-3′ for all constructs, and individual forward primers, 5′-TTTGGTACCGCCACCATGAGAGAAAAAAGAGCAGTGGGAATAGG-3′ (for pIg-gp41ect and pIg-gp41ect-L568P); 5′-TTTGGTACCGCCACCA TGGCAACGGTACAGGCCAGACAAT-3′ (for pIg-gp41ect$_{-\Delta FP}$ and pIg-gp41ect-L568P$_{-\Delta FP}$); 5′-TTTGGTACCGCCACCATGGAATTATTGGAATT AGATAAATGG-3′ (for pIg-MPER). Single- and double-underlined sequences indicate restriction-enzyme sites of BamHI and KpnI, respectively. Amplified DNA fragments were digested with KpnI and BamHI and ligated into corresponding sites of vector pIg-tail (R&D Systems, Inc.). The sequences of all constructs were confirmed by DNA sequencing.

Circular dichroism (CD) spectroscopy. CD spectra of NCwt and NC(L568P) were acquired on Jasco spectropolarimeter (Model J-715, Jasco Inc., Japan) at 20 °C using a 50 nm band with 1 nm resolution, and 0.1 cm path length. The final concentration of each protein is 10 nM in PBS. The CD spectra of samples were corrected by subtraction of the PBS background solvent spectrum. Thermal stability was monitored at 222 nm by applying a thermal gradient of 2° intervals from 20 °C to 98 °C.

Transient transfections. All constructs were transfected into CHO cells. One day prior to the transfection, 4×10^5 cells in DMEM containing 10% FBS and 1% penicillin–streptomycin (pen–strep) were seeded in a 60-mm tissue culture dish. These cells were transfected with the plasmids encoding recombinant gp41 ectodomain at 50–70% confluency using Vigofect kit (Vigorous, Pekin, PRC). One day after transfection, cells were collected for analysis.

Flow cytometry. The CHO cells transfected with various plasmids were analyzed by flow cytometry. CHO cells were harvested with dissociation buffer (0.5 mM EDTA + 0.5 mM EGTA in PBS, pH 7.0), centrifuged at 1000 rpm for 3 min, and re-suspended at 4×10^6 cells/ml. The cells were incubated with primary antibodies at RT for 1 h and then washed three times with PBS containing 2% fetal bovine serum (FBS). FITC-conjugated goat anti-mouse IgG was added at 1:40 dilution and incubated for 40 min at RT, followed by three washes. The cells were then re-suspended in PBS containing 2% FBS and analyzed with a Becton Dickinson fluorescence-activated cell sorter LSR II (BD Biosciences, San Jose, CA) at 10,000 events/sample with respect to unlabeled cells. The data were analyzed using CellQuest (Becton Dickison, Sunnyvale, USA).

Mouse immunizations and production of murine anti-4E10 epitope Abs. BALB/c mice (five mice for each sample) were immunized four times intramuscularly with 100 μg of an eukaryotic expression plasmid encoding a region of gp41 ectodomain. Four days after the last immunization, serum samples were collected for determination of antibody titers.

Direct ELISA. A standard ELISA was used to determine antibody binding to the peptide 4E10P2. Briefly, high-binding 96-well microtiter plates (Greiner, Ntirtingen, Germany) were coated with 4E10P2 in carbonate–bicarbonate buffer (pH 9.6) at 5 μg/well, overnight at 4 °C. Plates were blocked with PBS containing 0.25% (wt./vol) gelatin for 2 h at room temperature. All sera were diluted in the same blocking buffer. Then horseradish peroxidase-labeled goat anti-mouse IgG (Dako A/S, Denmark) and the substrate were added sequentially. The absorbance at 490 nm (A490) was measured in an ELISA reader (Bio-Rad, USA).

Results

Design and construction of eukaryotic expression plasmids encoding gp41 ectodomain and its fragments fused with IgG Fc

In this study, we adopted a pIg-tail expression system, which can fuse the Fc-tail of IgG to target proteins, to express gp41 ectodomain and its fragments on surface of cell membrane. To confirm whether this pIg-tail vector could effectively transport proteins to the membrane surface, we constructed an enhanced-GFP (eGFP) pIg-tail fusion plasmid which was transfected into CHO cells. Confocal microscopic analysis demonstrated that the green fluorescence products of pIg-eGFP distributed primarily around cell membrane (Fig. 1A), while the product of pcDNA3.0-eGFP are diffused evenly in whole cell (Fig. 1B).

Then we used pIg-tail expression system to construct a panel of eukaryotic expression plasmids that encode gp41 ectodomain with or without FP (Fig. 2A). We also introduced a point mutation (L568P) in NHR that may disrupt the gp41 6-HB conformation [17,18]. SDS–PAGE analysis revealed the expressed proteins of NCwt and NC(L568P) with expected molecular weight (Fig. 2B). At 20 °C, both NCwt and NC(L568P) displayed helical structure with similar α-helicity (Fig. 2C). However, the α-helicity of NC(L568P) reduced tremendously as the temperature rose and no typical melting temperature (T_m) could be determined for this protein, while NCwt is highly thermal stable with a T_m of 84 °C (Fig. 2D). These results confirmed that L568P mutation remarkably destabilizes the conformation of the 6-HB formed by NHR and CHR.

The effect of FP deletion and/or L568P mutation on the antigenicity of the 4E10 epitope

We transfected equal amount of DNA of each plasmid into CHO cells and analyzed the binding of 4E10-epitope-specific antibodies to those cells by flow cytometry. 4E10 antibodies showed higher binding to cells transfected with pIg-MPER than to those transfected with pcDNA3.0-MPER (Fig. 3A), indicating that pIg-tail vector could present 4E10 epitopes on cell surfaces. However, the antibody binding to the cells transfected with pIg-gp41ect plasmid, which encodes the intact ectodomain of gp41, was significantly reduced (Fig. 3B). Then, we determined the impact of FP deletion

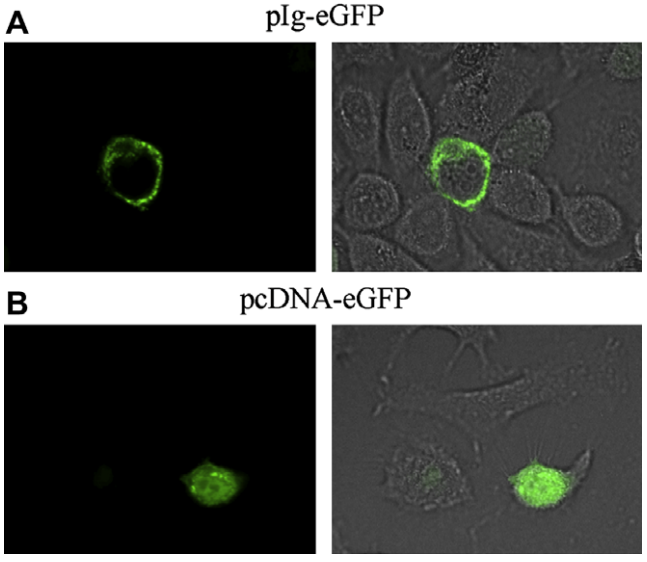

Fig. 1. Localization of eGFP in CHO cells transfected with (A) pIg-eGFP and (B) pcDNA-eGFP.

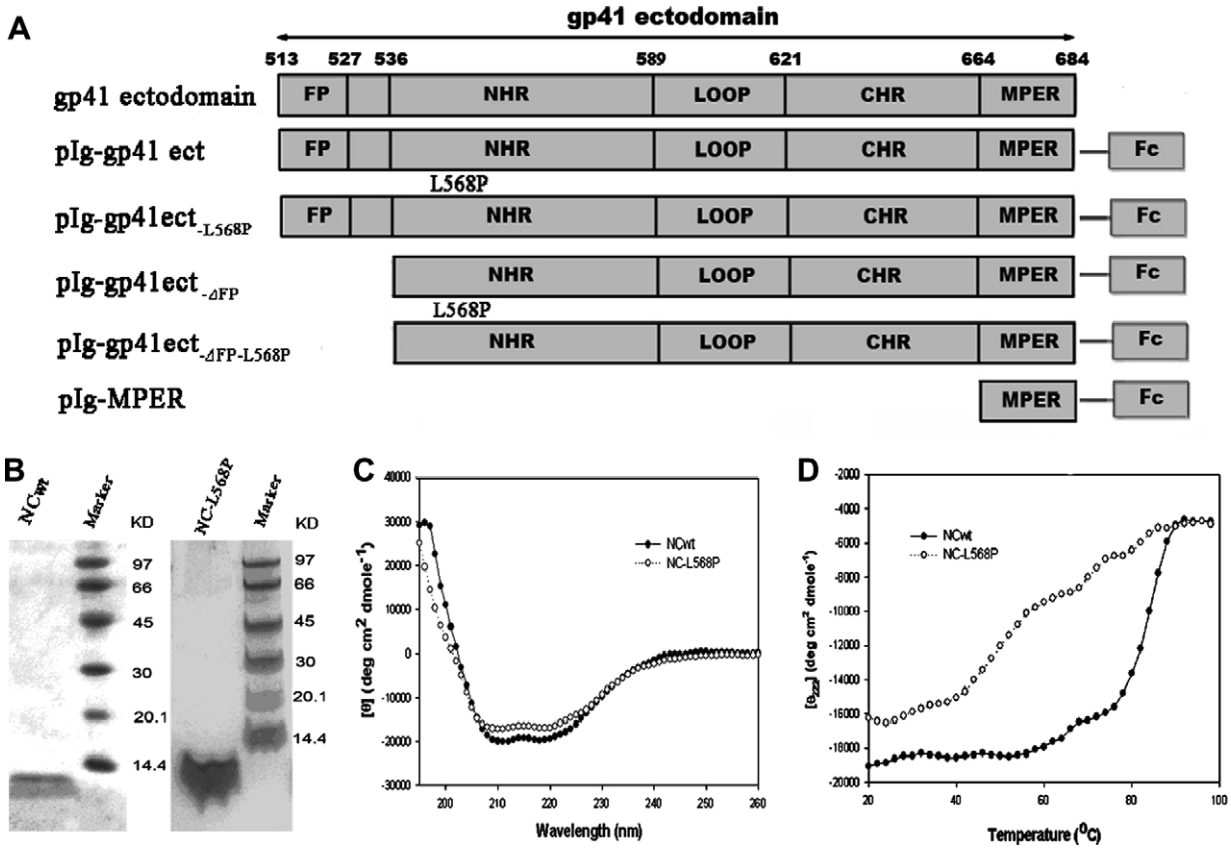

Fig. 2. (A) Schematic representation of the constructs encoding gp41 ectodomain and its fragments fused with IgG Fc tail. (B) SDS–PAGE for analysis of the expressed NCwt and NC(L568P). (C) CD spectra of NCwt and NC(L568P). (D) Thermal stability which was monitored at 222 nm by applying a thermal gradient of 2° intervals from 20 °C to 98 °C.

and/or L568P mutation on the antigenicity of 4E10 epitope. As shown in Fig. 3C, L568P mutation in the gp41 NHR resulted in significant enhancement of antibody binding to the 4E10 epitope, suggesting that disruption of the 6-HB conformation caused by the L568P mutation may enhance the antigenicity of the 4E10 epitope. In addition, the existence of FP may have a negative effect on the accessibility of 4E10 epitope because the binding of antibodies with 4E10 epitope on cell membranes was obviously enhanced after deleting the gp41 FP (Fig. 3D).

The effect of FP deletion and/or L568P mutation on the immunogenicity of the 4E10 epitope

To determine the immunogenicity of 4E10 epitope in the gp41 MPER with FP deletion and/or L568P mutation, we immunized mice with those eukaryotic plasmids as DNA vaccines. After immunizing four times, we collected the antisera for measuring their titers against the peptide 4E10P2 by ELISA. As shown in Fig. 4, the antisera of mice immunized with pcDNA-MPER and pIg-MPER showed similar titers of antibodies. Notably, the titers of antisera of mice immunized with intact ectodomain of gp41 were even lower than that of the above two. These data were consistent with our FACS data, i.e., intact ectodomain of gp41 might shield the 4E10 epitope. Interestingly, the sera against both NHR mutants, pIg-gp41ect$_{-L568P}$ and pIg-gp41ect$_{-\Delta FP-L568P,}$ had a relatively higher affinity to the peptide 4E10P2, compared with that of pIg-MPER (Fig. 4). These findings suggest that the L568P mutation-induced disruption of the gp41 core may not only favor a better accessibility of 4E10 epitope on lipid membranes, but also improve the immunogenicity of this epitope. On the other hand, FP region seems to have little effect on the immunogenicity of this epitope, since the titers of antisera against pIg-MPER and pIg-gp41ect$_{-\Delta FP}$ exhibited no significant difference.

Discussion

Although the HIV-1 gp41 is relatively conserved by comparison of gp120, only a few broadly neutralizing antibodies (BNAbs) against gp41 have been identified so far. Those BNAbs, including 2F5 and 4E10, all target the gp41 MPER, which contains a large number of hydrophobic residues with high lipid-membrane-related feature [14,20,21]. Previous studies have shown that 4E10 epitope in the lipid environment forms complicated conformation. Therefore, during natural infection, this epitope may be shielded, or difficult to access, or transiently exposed [22–26]. Due to lack of native conformation, the peptides containing the MPER sequence have failed to elicit neutralizing Abs. Thus, the gp41 MPER must maintain a proper conformation in order to elicit effective neutralizing antibodies.

In this study, we have used the pIg-tail system to express the gp41 ectodomain and its fragments with or without FP on the surface of cell membranes. We found that deletion of FP resulted in significantly increased antigenicity of 4E10 epitope (Fig. 3D), possibly because FP and MPER, both consisting of mainly hydrophobic residues, may interact with each other, resulting in obscuration of the 4E10 epitope in MPER.

We observed that the L568P mutation in the NHR, which may destabilize the gp41 6-HB core conformation [17,18], also significantly enhanced the antigenicity of 4E10 epitope (Fig. 3C). Dimitrov et al. have shown that the binding of 4E10 decreased upon

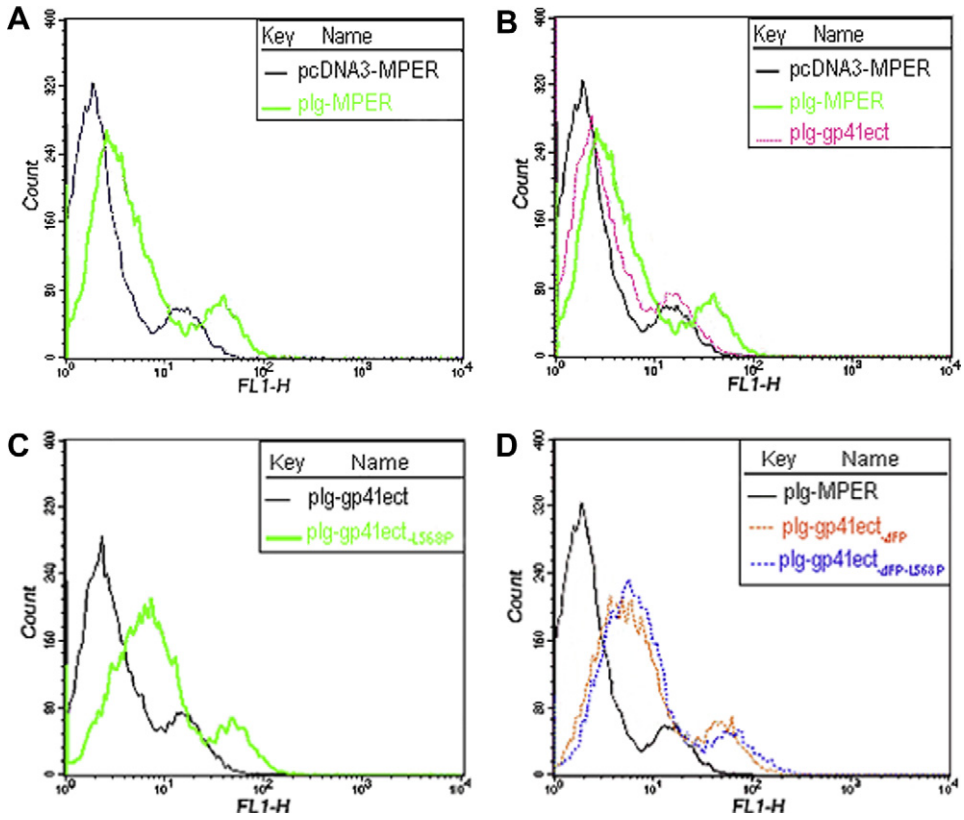

Fig. 3. 4E10 epitope exposure on CHO cell surface measured by FACS. (A) Antibody binding to cells transfected with pcDNA3-MPER and pIg-MPER. (B) Antibody binding to cells transfected with pIg-MPER and pIg-gp41ect. (C) Antibody binding to cells transfected with pIg-gp41ect and pIg-gp41ect-$_{L568P}$. (D) Antibody binding to cells transfected with pIg-gp41ect-$_{\Delta FP\text{-}L568P}$ and pIg-gp41ect-$_{\Delta FP}$.

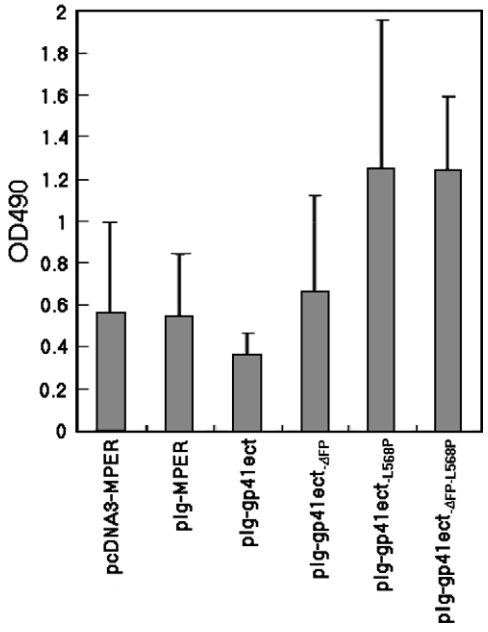

Fig. 4. Reaction of DNA vaccine-immunized sera with the peptide 4E10P2 by ELISA. All of detected antisera came from the last-immunized mice and analyzed at 1:100 dilution.

the gp41 6-HB formation triggered by interaction of the target cells with the HIV-1 Env-expressing cells [27]. Blish et al. have reported that a natural mutation (T569A) in gp41 NHR results in increased neutralization sensitivity of the HIV-1 variant to 4E10 and 2F5 [28]. Since T569 is an important component of the gp41 hydrophobic cavity, which plays a critical role in the stabilization of the hetero-trimeric coiled coils [2,29], the T569A mutant may act in a similar way as the L568P mutant to disturb the conformation stability of 6-HB. These findings suggest that formation of the gp41 6-HB core may mask the 4E10 epitope, leading to the decreased antigenicity of this epitope.

Subsequently, we determined whether these increased exposures of the neutralization epitope would enhance the immunogenicity of 4E10 epitope by immunizing mice with these plasmids as DNA vaccines and comparing their antibody responses against the peptide containing the 4E10 epitope. We found that deletion of FP did not enhance the immunogenicity of the 4E10 epitope. However, the L568P mutation in NHR resulted in increased antibody response against 4E10 epitope.

Overall, we have found that deletion of FP from the gp41 ectodomain expressed on cell surface results in significant increase of the antigenicity of the 4E10 epitope, but does not affect its immunogenicity. However, L568P mutation in NHR leads to remarkable enhancement of the antigenicity and immunogenicity of the 4E10 epitope. These results suggest that alternation of the gp41 structure and conformation may affect the accessibility of the neutralizing epitopes in the gp41 MPER. Therefore, this information may be useful for rational design of effective vaccines targeting the HIV-1 gp41.

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