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Structure and Immunogenicity of a Peptide Vaccine, Including the Complete HIV-1 gp41 2F5 Epitope

IMPLICATIONS FOR ANTIBODY RECOGNITION MECHANISM AND IMMUNOGEN DESIGN*

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Background: HIV-1 vaccines should elicit broadly neutralizing antibodies as the gp41 "membrane-proximal external region" targeting MAb2F5.

Results: NMR disclosed unprecedented 2F5 peptide-epitope structures. Although overall conformation was preserved in different adjuvants, recovered antibodies after vaccination were functionally different.

Conclusion: Membrane-inserted helical oligomers may encompass effective 2F5 peptide vaccines.

Significance: Disclosing the structures that generate 2F5-like antibodies may guide future vaccine development.

The membrane-proximal external region (MPER) of gp41 harbors the epitope recognized by the broadly neutralizing anti-HIV 2F5 antibody, a research focus in HIV-1 vaccine development. In this work, we analyze the structure and immunogenic properties of MPERp, a peptide vaccine that includes the following: (i) the complete sequence protected from proteolysis by the 2F5 paratope; (ii) downstream residues postulated to establish weak contacts with the CDR-H3 loop of the antibody, which are believed to be crucial for neutralization; and (iii) an aromatic rich anchor to the membrane interface. MPERp structures solved in dodecylphosphocholine micelles and 25% 1,1,1,3,3,3-hexafluoro-2-propanol (v/v) confirmed folding of the complete 2F5 epitope within continuous kinked helices. Infrared spectroscopy (IR) measurements demonstrated the retention of

main helical conformations in immunogenic formulations based on alum, Freund's adjuvant, or two different types of liposomes. Binding to membrane-inserted MPERp, IR, molecular dynamics simulations, and characterization of the immune responses further suggested that packed helical bundles partially inserted into the lipid bilayer, rather than monomeric helices adsorbed to the membrane interface, could encompass effective MPER peptide vaccines. Together, our data constitute a proof-of-concept to support MPER-based peptides in combination with liposomes as stand-alone immunogens and suggest new approaches for structure-aided MPER vaccine development.

The envelope (Env)⁸ glycoprotein subunits gp120 (surface) and gp41 (transmembrane), which mediate receptor binding and virus-cell fusion, respectively, are organized as trimers of noncovalently associated heterodimers on the surface of the HIV-1-producing cells and assembled virions (1, 2). Upon receptor/co-receptor engagement by gp120, the gp41 ectodomain undergoes a series of conformational changes to deliver the energy required for membrane merger (3–5). The functional Env complex is also targeted by the broadly neutralizing antibodies (bNAbs) known to block infection by a wide range of HIV-1 strains (1, 4, 6). These antibodies are triggered in a

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† This article contains supplemental Tables S1 and S2.

The atomic coordinates and structure factors (codes 2M8M and 2M8O) have been deposited in the Protein Data Bank (<http://www.pdb.org/>).

¹ H chemical shifts for MPERp in HFIP and DPC were deposited in the BioMagResBank under accession number 19262 and 19263.

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⁸ The abbreviations used are: Env, envelope; bNAbs, broadly neutralizing antibody; Chol, cholesterol; DPC, dodecylphosphocholine; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; MPER, membrane-proximal external region; PA, phosphatidic acid; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol; TMD, transmembrane domain; PDB, Protein Data Bank; CDR, complementarity-determining region; MDS, molecular dynamics simulation.

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fraction of infected individuals only upon prolonged contact with the virus (7, 8). It has been proposed that vaccination strategies focused on the induction of antibodies qualitatively similar to those bNAbs might result in the effective prevention of infection (7, 9).

The isolation of bNAbs in the form of monoclonal antibodies (MAbs) has revealed common structural trends useful for guiding the rational design of immunogens eliciting protective antibodies (9–11). Broad neutralization is attained by antibodies that bind to a handful of invariable but accessible regions of gp120 and gp41. Broadly neutralizing sera raised to gp120 have been found to contain antibodies that target the receptor-binding site, the glycan-V3 site, and the V1V2 loops, whereas antibodies in broadly neutralizing sera raised to gp41 appear to bind exclusively to the “membrane-proximal external region” (MPER) or pre-transmembrane domain within this subunit (7–10, 12, 13).

In contrast to the structurally complex, discontinuous epitopes recognized by anti-gp120 bNAbs, it is hypothesized that MPER embodies a single continuous linear epitope (14–18). Following this idea, it has been suggested that synthetic peptides constrained into the neutralization-competent MPER structures might constitute stand-alone vaccines (19, 20). One anti-MPER bNAb that has focused much attention in this research area is the 2F5 antibody. 2F5 was isolated in mAb form by Katinger and co-workers (21, 22) from a panel of sera from naturally infected asymptomatic individuals. Given the neutralization breadth and potency shown by the bNAb 2F5 (13, 21, 23–26), development of peptide-based vaccines targeting the 2F5 epitope has since been pursued (6, 22, 27–33).

Binding specificity of MAb2F5 was initially mapped to N-terminal ⁶⁶²ELDKWA⁶⁶⁷ MPER residues (21, 24, 26). Based on mass spectrometry and proteolytic protection assays, this core epitope was later extended to span the ⁶⁵⁶NEQELLELDK-WASLWN⁶⁷¹ sequence (34). Comparable full epitope lengths were subsequently suggested by competition ELISA (35) and structural analyses (14, 36). X-ray crystallography further indicated that epitope binding does not involve the hydrophobic apex of the long complementarity-determining region (CDR)-H3 loop, an element shown to be crucial for the neutralizing function of the antibody (37, 38). Given the close proximity of the epitope to the envelope surface, it has been proposed that the 2F5 CDR-H3 loop might interact directly with viral membrane lipids (14, 39–41). Alternatively, data have been recently reported suggesting that the CDR-H3 loop apex may establish additional contacts with MPER C-terminal residues in helical conformation (25, 38). These two options need not be mutually exclusive for bivalent antibodies targeting the 2F5 epitope on the surface of virions. It has been argued that MAb2F5-like antibodies could use a heterologation strategy (*i.e.* to combine strong binding to gp41 and weak binding to viral membrane) to increase its avidity under conditions existing in the HIV envelope (9).

Here, we provide unprecedented results on the structure and immunogenicity of a peptide spanning the sequence ⁶⁵⁶NEQELLELDKWASLWNWFNITNWLWYIK⁶⁸³, which includes the complete 2F5 epitope (underlined), the downstream region proposed to establish weak contacts with the CDR-H3 loop of

the antibody, and an aromatic-rich block that allows its insertion into the membrane interface (Fig. 1). The NMR data on this peptide, termed MPERp, support the folding of the complete HIV-1 2F5 epitope within a continuous kinked helix.

IR confirmed the preservation of the main helical conformation in adjuvants representing licensed vaccine formulations (*i.e.* aluminum salt and water-in-oil emulsions) and in two different types of liposomes. Because it is predicted that the liposomal MPERs that mimic the 2F5 epitope will be bound by the functional neutralizing antibody, we performed assays to correlate function and binding. Consistent with previous reports (37, 38), cell infection blocking in our in-house assay was dependent on the CDR-H3 loop. 2F5 binding to MPERp in liposomes made of anionic phospholipid and lipid A was also dependent on the CDR-H3 loop, whereas binding to the peptide on the surface of lesser charged Chol-containing vesicles did not require this element.

All tested MPERp vaccines were immunogenic. However, significant amounts of 2F5 epitope-targeting antibodies with the capacity of blocking cell infection were only recovered from sera of rabbits immunized with liposomal vaccines displaying a correlation between 2F5 antibody function and binding, *i.e.* those based on the anionic phospholipid and lipid A. Insights into the structural basis for functional antibody generation could be gained by combining IR and molecular dynamics simulation (MDS) analyses. These data suggest that membrane-inserted helical bundles, rather than monomers adsorbed to the membrane interface, may embody efficient MPER vaccines. Together, our structural and immunogenicity data conform to the prediction that MPER may fold as a single contiguous antigenic determinant, competent in generating a neutralizing response and therefore supporting the application of derived peptides in combination with liposomes as stand-alone vaccines to target the 2F5 epitope.

EXPERIMENTAL PROCEDURES

Materials—MPERp and the 2F5 peptide epitopes used in the immunological studies were synthesized in C-terminal carboxamide form by solid phase methods using Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry, purified by reverse phase HPLC, and characterized by matrix-assisted time-of-flight (MALDI-TOF) mass spectrometry (purity >95%). Peptides were routinely dissolved in dimethyl sulfoxide (DMSO, spectroscopy grade), and their concentration was determined by the bicinchoninic acid microassay (Pierce). 1-Palmitoyl-2-oleoylphosphatidylglycerol (POPG), 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), phosphatidic acid (PA), Chol, and lipid A detoxified (*Salmonella minnesota* R595) were purchased from Avanti Polar Lipids (Birmingham, AL). Dodecylphosphocholine (DPC) was from Anatrace (Maumee, OH). MAb2F5 was kindly donated by Dietmar Katinger (Polymun Scientific, Klosterneuburg, Austria). Fab2F5-WT and Fab2F5- Δ CDR-H3 were produced as described previously (38).

Recording of NMR Spectra—NMR samples were prepared by dissolving the lyophilized peptides (~1 mg) in 0.5 ml of a H₂O/D₂O (9:1 ratio by volume) solution containing 2 mM HEPES buffer at pH 7.0 and either 25% (v/v) 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP; D2, 98%; Cambridge Isotopes Lab) or 20 mM

TABLE 1

Structural statistics for the ensemble of the 20 lowest energy NMR structures of MPERp in DPC (20 mM deuterated dodecylphosphocholine, 2 mM HEPES, pH 7.0, H₂O/D₂O, 9:1, v/v) and HFIP (25% deuterated 1,1,1,3,3,3-hexafluoro-2-propanol in 2 mM HEPES, pH 7.0, H₂O/D₂O, 9:1, v/v)

		DPC	HFIP
No. of distance restraints	Intraresidue ($i - j = 0$)	154	118
	Sequential ($ i - j = 1$)	103	74
	Medium range ($1 < i - j < 5$)	115	35
	Total number	372	227
	Averaged total no./residue	13.3	8.0
No. of dihedral angle constraints	ϕ angles	26	26
	ψ angles	25	22
	Total number	51	48
	Average maximum violations/structure	Distance (Å)	0.15 ± 0.01
Averaged structure energies	Dihedral angle (°)	3.5 ± 0.1	0.1 ± 0.1
	CYANA target function value	0.35 ± 0.01	0.01 ± 0.01
	AMBER energy (kcal/mol)	-1187	-602
	van der Waals energy (kcal/mol)	-195	-182
	Electrostatic energy (kcal/mol)	-2247	-2235
Deviations from ideal geometry	Bond length (Å)	0.014	0.015
	Bond angle (°)	1.8	1.7
Pairwise root mean square deviation (Å) (residues 2–27)	Backbone atoms	0.4 ± 0.4	1.6 ± 0.7
	All heavy atoms	1.1 ± 0.3	2.5 ± 0.7
Ramachandran plot (%)	Residues in most favored regions	99.6	98.8
	Residues in additional allowed regions	0.4	1.2
	Residues in generously allowed regions	0.0	0.0
	Residues in disallowed regions	0.0	0.0

deuterated DPC (D38, 98%; Cambridge Isotopes Lab). Peptide concentrations were ~0.5 mM. pH was measured with a glass microelectrode and not corrected for isotope effects. A methanol sample was used to calibrate the temperature of the NMR probe. Chemical shifts were referenced to internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate.

The ¹H NMR spectra were acquired on a Bruker Avance-600 spectrometer operating at a proton frequency of 600.13 MHz and equipped with a cryoprobe. One-dimensional spectra were acquired using 32 K data points, which were zero-filled to 64 K data points before performing the Fourier transformation. Phase-sensitive two-dimensional correlated spectroscopy (COSY), total correlated spectroscopy (TOCSY), and nuclear Overhauser enhancement spectroscopy (NOESY) spectra were recorded by standard techniques using presaturation of the water signal and the time-proportional phase incrementation mode, as reported previously (42). NOESY mixing times were 100 or 150 ms, and TOCSY spectra were recorded using 60 ms DIPSI2 with z filter spin-lock sequence. Acquisition data matrices were defined by 2048 × 512 points in t_2 and t_1 , respectively. Data were processed using the standard TOPSPIN program (Bruker Biospin, Karlsruhe, Germany). The two-dimensional data matrix was multiplied by either a square-sine-bell or a sine-bell window function with the corresponding shift optimized for every spectrum and zero-filled to a 2K × 1K complex matrix prior to Fourier transformation. Baseline correction was applied in both dimensions.

Structure Calculation—The same protocol was followed to calculate the structures of MPERp in the presence of DPC micelles and in 25% HFIP from distance and dihedral angle constraints derived from NMR parameters. Distance constraints were obtained from the 150-ms two-dimensional ¹H-¹H NOESY spectra, with the cross-peaks observed in the 100-ms two-dimensional ¹H-¹H NOESY being essentially the same. Dihedral angle restraints for ϕ and ψ angles were derived from ¹H _{α chemical shifts using the program TALOS (43). Structures were calculated using a three-step protocol. First, the standard iterative procedure for automatic NOE assign-}

ment of the program CYANA 2.1 (44) was applied. The protocol consists of seven cycles of combined automated NOE assignment and structure calculation of 100 conformers per cycle (45). The list of distance constraints resulting from the last automatic cycle was checked by inspection of the corresponding NOESY spectra, and ambiguous constraints were removed or relaxed to generate the final list used as input for a standard simulated annealing CYANA 2.1 calculation of 100 conformers. The 20 conformers with the lowest target function values were selected and subjected to 2000 steps of energy minimization using the generalized Born continuum solvation model with a nonbonded cutoff of 10 Å as implemented in the program AMBER9 (Case DA, Darden TA, Cheatham III TE, University of California, San Francisco). The structural statistics data for the final ensembles of 20 structures obtained for MPERp are provided in Table 1. The quality of these final structures was assessed using PROCHECK/NMR (46) as implemented at the Protein Structure Validation Suite server. All of the residues were either in the most favored or allowed regions of the Ramachandran map (Table 1). The structural ensembles calculated for MPERp have been deposited in the PDB Data Bank with accession codes 2M8M (HFIP) and 2M8O (DPC). These structures were visualized and examined using the programs MOLMOL (47) and Swiss-Pdb viewer (48).

Membrane Binding Assays—Vesicle flotation experiments in sucrose gradients were performed following the method described by Yethon *et al.* (49). In brief, 100 μ l of a sample containing rhodamine-labeled liposomes (3 mM lipid concentration) was adjusted to a sucrose concentration of 1.4 M in a final volume of 300 μ l, and subsequently overlaid with 400- and 300- μ l layers of 0.8 and 0.5 M sucrose, respectively. The gradient was centrifuged at 436,000 × *g* for 3 h in a TLA 120.2 rotor (Beckman Coulter, Brea CA). After centrifugation, four 250- μ l fractions were collected. Material adhered to tubes was collected into a 5th fraction by washing with 250 μ l of hot (100 °C) 1% (w/v) SDS.

Infrared Spectroscopy—Infrared spectra were recorded in a Thermo Nicolet 5700 spectrometer equipped with a mercury-

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cadmium-telluride detector using a Peltier-based temperature controller (TempCon, BioTools Inc., Wauconda, IL) with calcium fluoride cells (BioCell, BioTools Inc., Wauconda, IL). MPERp-containing samples were lyophilized and subsequently prepared at 4 mg/ml in D₂O buffer (5 mM HEPES, pD 7.4, 100 mM NaCl). A 25- μ l sample aliquot was deposited on a cell that was sealed with a second cell. Reference windows without peptide were prepared similarly. Typically 370 scans were collected for each background and sample, and the spectra were obtained with a nominal resolution of 2 cm⁻¹. Data treatment and band decomposition of the original amide I have been described elsewhere (50).

Cell Entry Assays—For the in-house cell entry assays (38), HIV-1 pseudoviruses were produced by transfection of human kidney HEK293T cells with the full-length Env clone JRCSF (kindly provided by Jamie K. Scott and Naveed Gulzar, Simon Fraser University, British Columbia, Canada) using calcium phosphate. Cells were co-transfected with vectors pWXLPGFP and pCMV8.91, encoding, respectively, a green fluorescent protein and an Env-deficient HIV-1 genome (provided by Patricia Villace, Consejo Superior de Investigaciones Científicas, Madrid, Spain). After 24 h, the medium was replaced with OptiMEM-Glutamax II (Invitrogen) without serum. Two days after transfection, the pseudovirus particles were harvested, passed through 0.45- μ m pore sterile filters (Millex[®] HV, Millipore NV, Brussels, Belgium), and finally concentrated by ultracentrifugation in a sucrose gradient. HIV entry was determined using TZM-bl target cells (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health, contributed by J. Kappes). Antibody samples were set up in duplicate in 96-well plates and incubated for 1 h at 37 °C with a 10–15% tissue culture infectious dose of pseudovirus. After antibody-pseudovirus co-incubation, 10,000 target cells were added in the presence of 15 μ g/ml DEAE-dextran (Sigma). Infection levels after 72 h were inferred from the number of GFP-positive cells as determined by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences).

Molecular Dynamics Simulations—Atomic coordinates of the MPER peptide were taken from the NMR structure calculated in DPC micelles (first model). Only gp41 residues 656 to 683 were included in the model, with sequence NEQELLELDKWLWVFNITNWLWYIK. Default protonation states were used for all the ionizable residues. N and C termini were amidated and acetylated, respectively.

A pre-equilibrated bilayer containing a mixture of POPC/1-palmitoyl-2-oleoyl phosphatidic acid/Chol in ratios 2:1.5:0.2 and pure POPG in a one-component bilayer were used. The systems were solvated by ~37,000 water molecules. Sodium and chloride ions were added to neutralize the systems up to a final experimental concentration of 150 mM. Four MPER peptides were randomly placed in the solution at the start of the simulations. The total production runs were 235 ns for each simulation. MD trajectories were simulated with the version 2.9 of NAMD (51), using the CHARMM27 force field with CMAP corrections for the peptides (52), the CHARMM36 force field for lipids (53), the TIP3P model for water molecules (54), and the model of Cournia *et al.* (55) for cholesterol. Standard parameters for ions in the CHARMM27 force field were

adopted. Simulations were performed in the NpT ensemble. Pressure was kept at 1 atm by the Nose-Hoover Langevin piston method (56, 57) with a damping time constant of 100 ps and a period of 200 ps. Temperature was kept at 300 K by coupling to a Langevin thermostat, with a damping coefficient of 5 ps⁻¹ (57). Electrostatic interactions were treated by the Particle Mesh Ewald algorithm, with grid spacing below 1 Å (58). van der Waals interactions were truncated at 12 Å and smoothed at 10 Å. Hydrogen atoms were restrained by the SETTLE algorithm (59), which allowed a 2-fs time step.

Rabbit Immunization and Antibody Purification—For immunization in Freund's adjuvant or alum, MPERp was dissolved in 0.5 ml of PBS and mixed with an equal volume of 1.3% (w/v) aluminum hydroxide (Alhydrogel, Superfos Biosector, Denmark) or Freund's adjuvant (Sigma). Liposome-based formulations were prepared following the methods described by Dreesman *et al.* (60) and Maeso *et al.* (61) and included lipid A as adjuvant (62). MPERp in DMSO was added at a final peptide-to-lipid ratio of 1:50 (mol/mol) to a stirring solution of freeze-thaw POPC/Chol/PA/lipid A (2.0:1.5:0.2:0.01 molar ratio) or POPG/lipid A (3.7:0.01 molar ratio) vesicles dispersed in PBS. After incubation for 30 min, the samples were lyophilized. New Zealand White rabbits were inoculated intradermally at multiple sites on day 0 with 1 ml of sample reconstituted in pure water, which contained 0.5 mg of peptide. For subsequent boosting injections, 1 ml of the reconstituted liposome formulation containing 0.3 mg of peptide was used on day 15 (0.3 mg peptide), and 0.2 mg of liposomal peptide was injected on days 30, 45, and 60. The 2F5 epitope-specific antibodies were recovered from sera through affinity purification. To that end, the 2F5ep-Cys (NEQELLELDKWLWVFNITNWLWYIK) peptide was immobilized onto a beaded agarose support using a Sulfolink immobilization kit for peptides (Thermo Scientific, Rockford, IL) and following the manufacturer's instructions. The remaining nonspecific binding sites in columns were blocked adding L-cysteine-HCl at 50 mM. Every analyzed serum was loaded on the columns after diluting and filtering it to remove the particulate material. They were allowed to flow through the columns five times thus allowing the binding of all the antibodies present in the serum that recognize specifically the immobilized peptide. After washing the columns with at least 10 bed volumes of 500 mM NaCl containing buffer to dispose of nonspecifically bound antibodies and serum proteins, the specific antibodies were eluted using 100 mM glycine buffer at pH 2.5. The fraction that is not recovered using acidic pH was eluted using freshly made 100 mM triethylamine buffer at pH 11.5.

RESULTS

Designation of the MPER Peptide Containing the Complete 2F5 Epitope—The diagram displayed in Fig. 1A designates MPER as the membrane-proximal sequence that connects the gp41 globular ectodomain (FP-NHR-loop-CHR) with the membrane-spanning domain (TMD). Position for the core epitope recognized by the 2F5 bNAbs is also displayed. The organization of this region within the pre-fusion gp41 structure recognized by this antibody is presently unknown. Nonetheless, MPER is postulated to embody a single neutralization-competent structure (19, 20).

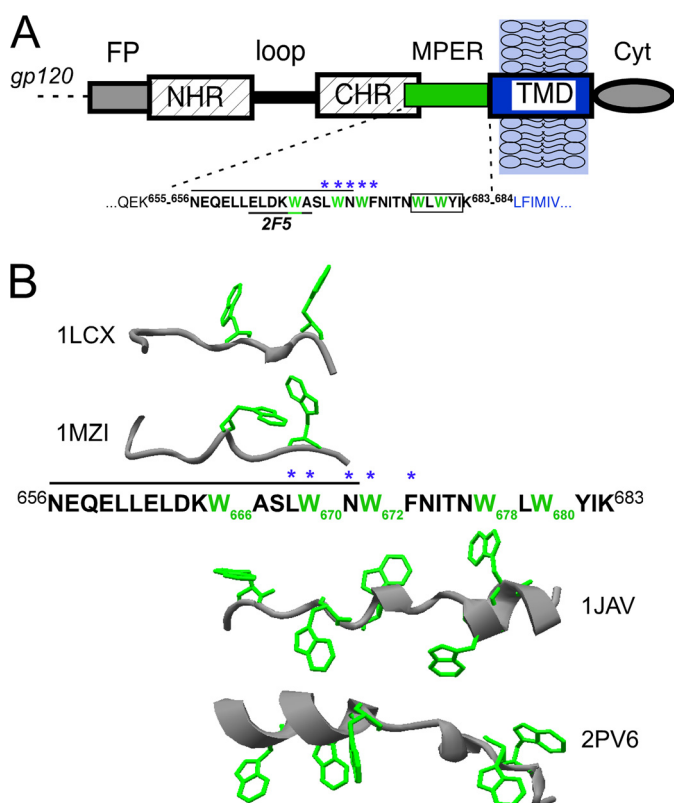


FIGURE 1. Design of MPER-derived peptide vaccine. *A*, scheme describing the HIV-1 gp41 organization and the sequence of the MPER peptide vaccine used in this study (HIV-1 Env residues 656–683, numbering and sequence derived from the prototypic HXBc2 isolate). The gp41 ectodomain regions designated in the top diagram include the following abbreviations: *FP*, fusion peptide; *NHR* and *CHR*, N- and C-terminal helical regions, respectively; *Cyt*, cytosolic domain. The MPER sequence below highlights the five Trp residues in green and the core epitope residues recognized by 2F5 antibody *underlined*. The *line* on top spans the extended 2F5 epitope as defined by proteomic analyses (34). *Blue asterisks* denote residues implied in secondary binding by CDR-H3 loop (25) and the *box* an aromatic rich anchor to the membrane interface. *B*, structures adopted by MPER-derived peptides. PDB accession numbers indicated in the panel designate structures in solution (1LCX and 1MZI) or in contact with DPC micelles (1JAV and 2PV6). Lateral side chains of Trp residues are depicted in *green* to align the structures with the MPER amino acid sequence.

Fine definition of the 2F5 epitope suggests the involvement in antibody recognition of a helical stretch that follows the core epitope residues (34, 36, 63). It has been argued that the MAb2F5's CDR-H3 loop establishes contact with residues located further downstream within a continuous helix (25, 38). Because the CDR-H3 loop is absolutely required for the 2F5 neutralizing activity (see below and Ref. 37), it is inferred that those residues constrained into a relevant conformation might be required for peptide vaccines to elicit broadly neutralizing 2F5-like antibodies. The reported three-dimensional NMR structures of MPER peptides are either disconnected from the MPER C-terminal residues (e.g. PDB codes 1LCX or 1MZI in Fig. 1*B*) or depleted from the N-terminal 2F5 epitope residues (e.g. PDB codes 1JAV or 2PV6 in Fig. 1*B*). Thus, we first attempted the NMR structure resolution of a synthetic peptide vaccine (MPERp, Fig. 1*B*), which included both a complete 2F5 epitope sequence and the C-terminal residues putatively engaged in secondary interactions with the CDR-H3 of MAb2F5 (25, 38).

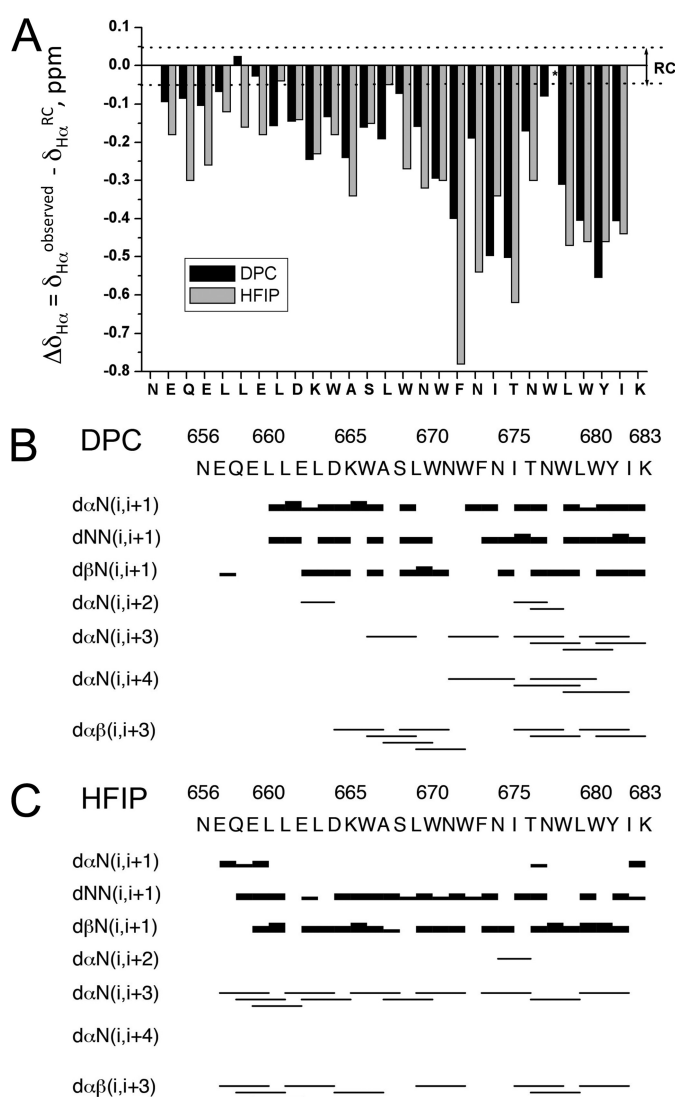


FIGURE 2. NMR parameters for MPERp. *A*, bar graphics showing the $\Delta\delta_{H\alpha}$ ($\Delta\delta_{H\alpha} = \delta_{H\alpha}^{\text{observed}} - \delta_{H\alpha}^{\text{RC}}$, ppm) values as a function of sequence in 20 mM DPC (*black bars*) or 25% HFIP (*gray bars*) at pH 7.0 and 25 °C. *Dashed lines* indicate the random coil (RC) ranges. Random coil values for $C_{\alpha}H$ protons were taken from Wishart *et al.* (66). The N- and C-terminal residues are excluded because of charged end effects. *B* and *C*, NOE summaries for the peptide in 20 mM DPC and 25% HFIP. The intensities of the sequential NOEs, classified as strong, medium, and weak, are indicated by the *thickness* of the *lines*.

NMR Solution Structure of MPERp—The 1H NMR signals of MPERp in the presence of DPC micelles and in 25% HFIP at pH 7.0 and 25 °C were assigned by standard sequential assignment methods (64, 65). These chemical shifts have been deposited at BioMagResBank with accession codes BMRB-19263 (DPC) and BMRB-19262 (HFIP). In this peptide, under both conditions, most 1H chemical shifts (*supplemental Tables S1 and S2*) deviated significantly from random coil values (66). In particular, most H_{α} protons showed large negative $\Delta\delta_{H\alpha}$ values (Fig. 2*A*). This is a clear indication that MPERp adopted helical structures under both conditions, because according to the well established empirical relationship between the $\Delta\delta_{H\alpha}$ and the ϕ and Ψ as in previous page 3 dihedral angles, positive and negative $\Delta\delta_{H\alpha}$ values are characteristic of β -strands and α -helices, respectively. Additional and stronger evidence for the adoption

2F5 Binding to Liposomal Vaccines—In addition to the standard adjuvants, MPERp was combined with liposomes to compose synthetic vaccines. It has been suggested that interactions with the membrane interface on the surface of liposomes may select for the MPER conformations that are relevant for the induction of specific antibodies (62), whereas polyreactivity with membrane lipids could increase the binding avidity of those antibodies (9). For this study, we selected two different lipid compositions. In one formulation MPERp was administered together with POPC/Chol/PA (2:1.5:0.2 mol/mol) liposomes, and in a second formulation the peptide was combined with vesicles made of the anionic phospholipid POPG. It is assumed that peptide structures relevant for the 2F5 epitope will be bound by the functional 2F5 antibody but not by their inactive mutants. Thus, to discriminate functional *versus* non-functional binding, we used CDR-H3 mutant Fabs that bind peptide epitopes in solution but are not neutralizing (Fig. 5).

Fig. 5A compares the capacity for inhibiting viral entry of the MAb2F5 and two derived Fabs, Fab2F5-WT and Fab2F5- Δ CDR-H3, with the latter representing a mutant with the CDR-H3 loop deleted (38). MAb2F5 and Fab2F5-WT inhibited pseudovirus cell infection in our assay (Fig. 5A, *black* and *blue* symbols, respectively), whereas Fab2F5- Δ CDR-H3 had no effect (*red* symbols). To determine a functional correlation with epitope binding, we next compared the capacity of these antibodies to bind the liposomal vaccines (Fig. 5, B–E). Experiments were set up for obtaining pure vesicles floating on the top fractions of sucrose gradients (Fig. 5B). These assays indicated quantitative MPERp incorporation into the POPC/Chol/PA and POPG liposomes (Fig. 5C). Results displayed in Fig. 5D further demonstrated that MAb2F5 could effectively bind to the membrane-inserted peptide epitopes in both types of liposomes.

TABLE 2

Band position, assignment, and % area of the components obtained after curve-fitting of IR spectra displayed in Fig. 4A

HFIP		DPC	
Band position ^a	Area ^b	Band position ^a	Area ^b
	%		%
1675 (β -Turns/ 3_{10} -helix)	11	1678/1665 (β -Turns/ 3_{10} -helix)	14
1652 (α -helix-buried)	66	1650 (α -Helix-buried)	66
1632 (α -helix-solvated)	20	1630 (α -Helix-solvated)	19
1615 (aggregation)	2	1611 (aggregation)	1

^a Wave numbers in cm^{-1} . The conformation assigned for each position is indicated below (50, 68).

^b The values have been rounded off to the nearest integer.

TABLE 3

Band position, assignment, and % area of the components obtained after curve-fitting of IR spectra displayed in Figs. 4B and 6, A and B

Alum		Freund's adjuvant		POPC/Chol/PA		POPG	
Band position ^a	Area (%) ^b	Band position ^a	Area (%) ^b	Band position ^a	Area (%) ^b	Band position ^a	Area (%) ^b
1680/1669 (β -Turns/ 3_{10} -helix)	11	1678/1665 (β -Turns/ 3_{10} -helix)	26	1685/1671 (β -Turns/ 3_{10} -helix)	26	1665 (3_{10} -Helix)	3
1653 (α -Helix-buried)	45	1651 (α -Helix-buried)	52	1652 (α -Helix-buried)	46	1654 (α -Helix-buried)	52
1635 (α -Helix-solvated)	19	1631 (α -Helix-solvated)	17	1637 (α -Helix-solvated)	13	1639 (α -Helix-solvated)	39
1620/1693 (Aggregation)	25	1617 (Aggregation)	5	1615/1627/1686 (Aggregation)	15	1628 (Aggregation)	5

^a Wave numbers are in cm^{-1} . The conformation assigned for each position is indicated below (50, 68).

^b The values have been rounded off to the nearest integer.

Thus, according to this sole criterion, both types of vesicles containing peptide might encompass effective 2F5 immunogens. However, the comparison of the Fabs revealed different patterns (Fig. 5D). The functional Fab2F5-WT reproduced the binding pattern of the mAb (Fig. 5D, *top*). In contrast, the nonfunctional Fab2F5- Δ CDR-H3 could bind effectively to POPC/Chol/PA-MPERp liposomes but not to POPG-MPERp liposomes (Fig. 5D, *bottom*).

Thus, cell entry inhibition and binding to POPG-MPERp liposomes were both dependent on the CDR-H3 loop. Together, these results allowed establishing a correlation between function and binding to POPG-bound MPERp, which was not found for the POPC/Chol/PA-MPERp liposomes. Accordingly, we inferred that when used as immunogens POPG-MPERp vaccines would be more selective than POPC/Chol/PA-MPERp vaccines in activating 2F5-like B-cell receptors.

MPERp Structure in Liposomal Vaccines—To gain insights into the membrane-associated structures at the origin of the function-binding correlation, we carried out a combined infrared spectroscopy-molecular dynamics simulation study (Fig. 6). Samples of MPERp in contact with POPC/Chol/PA liposomes closely reproduced the IR absorption spectrum measured in the Freund samples (Fig. 6A, *left*). This finding is consistent with the comparable structures adopted by membrane-binding peptides at interfaces of reverse micelles and membranes (71). In sharp contrast, the amide-I band measured in the POPG-MPERp samples was narrower, suggestive of higher conformational order, and corresponded almost exclusively to α -helical conformers (Fig. 6B, *left*). In these samples, the maximum shift of the low frequency α -helix band and its increase in intensity were consistent with the existence of packing interactions between solvated helices (67, 68).

Further insights into the membrane-associated structures causing these spectral differences were obtained from the dynamics of the MPERp DPC structure simulated in the presence of POPC/Chol/PA and POPG lipid bilayers (Fig. 6, *right panels*). MPERp was observed to associate as a single monomer with the POPC/Chol/PA membrane (Fig. 6A, *right*). In the course of the simulation, this peptide monomer was observed to contact first with the membrane surface through the hydrophobic C-terminal side, and the amphipathic N terminus was inserted later. At the end of the simulation (235 ns), the peptide adopted a continuous helix, whose main axis was parallel to the lipid bilayer plane. In this state, the membrane interface-embedded side chains made contact preferentially with the polar headgroups of POPC, but not with Chol or PA. In addition, 2F5

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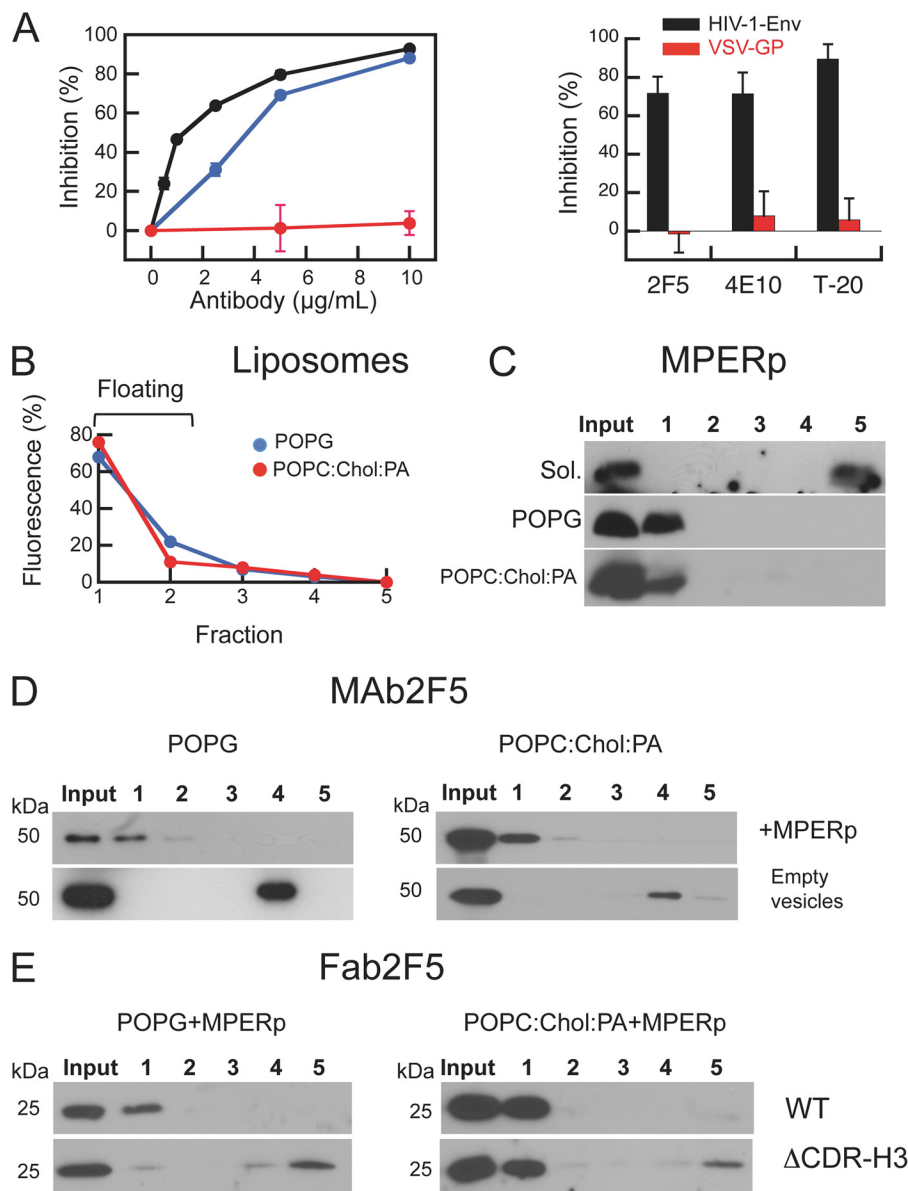


FIGURE 5. Correlation between 2F5 antibody function and binding to liposomal vaccines. *A*, cell entry inhibition assay. *Left*, pseudoviruses were preincubated with MAb2F5 or the recombinant 2F5 Fab constructs, and single cell entry events were monitored by FACS after incubation with TZM-bl target cells. Fab2F5 WT inhibited cell entry (*blue*), albeit with lower potency than the bi-functional mAb (*black*). In contrast Fab2F5 Δ CDR-H3 was almost completely unable to inhibit the process (*red*). *Right*, displays specificity controls for the HIV-1 Env-mediated cell entry. bNAbs (2F5 and 4E10) and T-20 were applied at 2 and 50 μ g/ml, respectively. Means \pm S.D. of six measurements in three independent experiments are displayed. *B–E*, vesicle flotation experiments in sucrose gradients. Rhodamine-labeled liposomes were collected in the 1st and 2nd fractions (*i.e.* floating fractions) (*B*). *C*, MPERp (30 μ M) was incubated in solution in absence (*top*) or presence of liposomes (peptide-to-lipid ratio of 1:100, *bottom panels*) for 15 min before centrifugation. The presence of the peptide in the different fractions was probed with MAb2F5 in Western blot. Virtually all input peptide co-floated with liposomes indicating quantitative partitioning into membranes. *D*, MAb2F5 (15 μ g ml⁻¹) was incubated for 15 min with MPERp-containing or empty liposomes before centrifugation (*top* and *bottom panels*, respectively). Consistent with antibody binding to membrane-inserted MPERp, MAb2F5 was recovered from the floating fractions upon incubation with peptide-containing liposomes. Similarly, Fab2F5 WT co-floated with both types of MPERp-containing liposomes (*E*, *top panels*). In contrast, in the case of POPG-MPERp vesicles, Fab2F5 Δ CDR-H3 was predominantly recovered from pellets (*E*, *bottom panels*).

epitope key residues Lys-665 and Trp-666 (Fig. 6A, *red side chains*), as well as residues establishing putative contacts with the CDR-H3 loop (*i.e.* Leu-669, Trp-672, and Phe-673) (*blue side chains*) were involved in these lipid interactions and therefore not accessible from the water solution.

In contrast, the four peptides, randomly placed in solution at the beginning of the simulation, were found assembled into a bundle at 194 ns. This bundle of peptides was associated with the POPG bilayer (Fig. 6B, *right*) from 194 ns until the end of the simulation (235 ns). The MPERp bundle inserted through the

aromatic rich C-terminal hydrophobic tip of one of the constituent monomers (Fig. 6B, residues in *green*) during the simulation. In the course of the simulation, distinct MPERp ensembles retaining overall helical conformation were observed several times to stick in the membrane by the action of a monomer; however, insertion of single peptides was not observed. Moreover, the more polar N terminus of the membrane-inserted monomer never contacted the negatively charged membrane surface. In the membrane-inserted state, residues involved in MAb2F5 recognition (Lys-665, Trp-666, Leu-669, Trp-672, and Phe-673 (see below and Refs. 25,

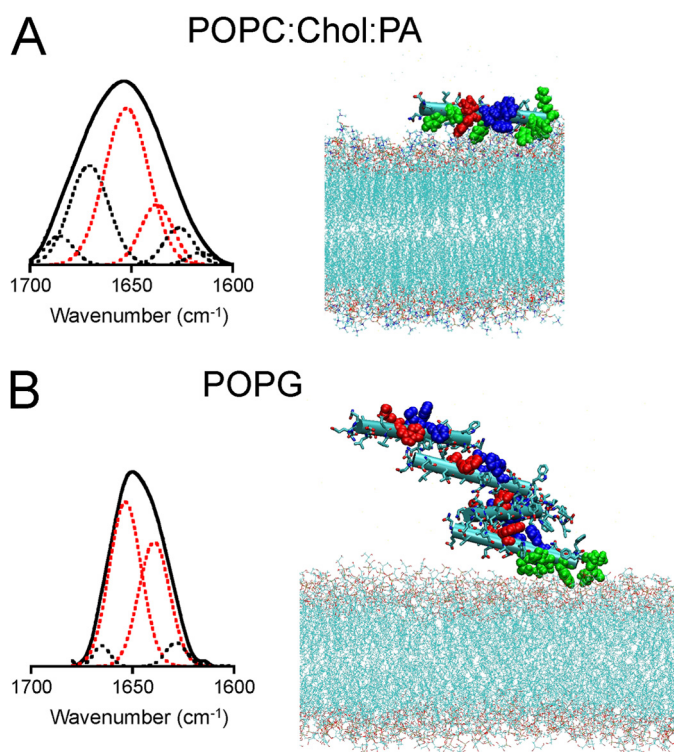


FIGURE 6. Combined IR and molecular dynamics simulations of MPERp interacting with POPC/Chol/PA (A) or POPG (B) lipid bilayers. *Left panels*, IR absorption in the amide I region by MPERp in D_2O -based buffer mixed with solutions of POPC/Chol/PA (2:1.5:0.2 mol/mol) liposomes or POPG liposomes, following the procedure used for preparing vaccines. In both panels, the IR spectra were decomposed into different band components (numerical values are disclosed in Table 3). *Red dotted lines* correspond to α -helix components. *Right panels*, snapshots of MPERp were taken at times 215 and 233 ns (*top* and *bottom*, respectively). Side views of the peptides display in space-filling representation residues Lys-665/Trp-666 in red and Leu-669/Trp-672/Phe-673 in blue. Phospholipids are shown in stick representation. Residues depicted in green have at least one atom within <3 Å from the phospholipid molecules.

36, 63) were all exposed to the solution. Nonetheless, the overall shape of the peptide ensemble restricted their accessibility from solvent in some of the monomers.

Immunogenicity of MPERp in the Different Adjuvants—To determine the immunogenicity of the structures described above, the MPERp-based alum, Freund's adjuvant, and liposome formulations were next compared in their capacity for activating B-cell responses (Fig. 7). Antigen-specific IgGs could be recovered upon immunization of rabbits with MPERp dispersed in alum or Freund's adjuvants (Fig. 7A, *left* and *left-center panels*). The midpoint titers in these samples were on the order of 10^3 . The responses triggered by the peptide-liposome formulations were weaker by comparison, showing midpoint titers in the order of 10^2 (Fig. 7A, *right-center* and *right panels*). Nonetheless, antibodies raised against the 2F5 epitope could be recovered from all sera through binding affinity (Fig. 7B). Supporting the same range of affinity, incubation in solution of the purified antibodies with 2F5 peptide epitope inhibited with comparable potencies their binding to MPERp deposited on plates (IC_{50} values in the order of $10 \mu M$ in all cases). In contrast, the experiments displayed in Fig. 7C indicated that the 2F5-targeting antibodies raised by the different formulations were qualitatively different. Those experiments revealed that the

only detectable inhibition of HIV entry occurred with the POPG/lipid A-MPERp formulation (Fig. 7C, *right-hand panel*). Of note, blocking VSV-GP-mediated cell infection was not observed in these experiments, which underscores the specificity of the recovered antibodies.

The inhibitory activity of these antibodies was nonetheless ~ 50 – 100 times lower than that displayed by the MAb2F5 in the cell entry assays (Fig. 5A). Thus, to establish the significance of this activity, we extended our studies on the immunogenicity of the POPG/lipid A-MPERp formulation (Fig. 8). Fig. 8A compares midpoint titers and cell entry inhibition by the 2F5-specific antibodies isolated from the sera of four different rabbits. These two values did not correlate, thereby suggesting that even if the immunogenicity levels were different, antibodies were functional in the cell entry assay after isolation in affinity columns. Two groups of sera, R2/R3 and R1/R4, were established as a function of the inhibitory strength of the isolated antibodies (Fig. 8B). For the two groups, the inhibition levels of HIV-Env-mediated cell entry were significantly higher than those in the VSV-GP controls. Thus, according to our data in Figs. 7 and 8, we may conclude that antibody samples recovered from POPG sera bore an inhibitory activity of cell entry that was not found in the samples recovered from alum, Freund's adjuvant, or POPC/Chol/PA sera.

DISCUSSION

It has been hypothesized that the membrane-proximal sequence connecting the gp41 subunit's ectodomain with the transmembrane anchor, termed the MPER domain, includes a continuous epitope. This implies that peptides recreating its native structure might in principle compose stand-alone HIV vaccines (6, 13, 19, 20, 72, 73). Although intensively studied, data supporting the structural MPER connectivity within a synthetic peptide vaccine were lacking. Here, we considered the incorporation within a single peptide of the sequence $^{656}NEQELLELDKWASLWN^{671}$ spanning the full epitope recognized by the MAb2F5 as defined by proteomic analyses, competition ELISA, and crystallography (14, 34–36), plus the following $^{672}WFNITNWLWYIK^{683}$ Trp-rich stretch that precedes the TMD (Fig. 1) (74). Our NMR data revealed the structuring of the resulting MPERp synthetic surrogate as a continuous helical structure (Figs. 2–4A). Structures of shorter or even longer peptides show spots of partial structuring as 3_{10} - and α -helix (33, 35, 73, 75–77), but none of them display continuous helical structures for the sequence spanning the full 2F5 epitope plus the downstream aromatic rich sequence preceding the TMD anchor (Fig. 1). This suggests that inclusion of the complete sequence covered by the 2F5 paratope (34) might be required for the long range interactions sustaining MPER folding as a continuous helix.

Although continuously helical, MPERp NMR structures solved in HFIP and DPC showed features implying a certain degree of conformational flexibility. The kink adopted by the $^{675}FNI^{677}$ residues at the C terminus of the DPC structure was consistent with the $^{673}FN^{674}$ hinge described previously for a shorter peptide (73). By comparison, the hinge of the shorter peptide induced a more abrupt change in backbone main direction and adopted a looser conformation (PDB code 2PV6, Fig.

Structure Immunogenicity of the Complete 2F5 Epitope

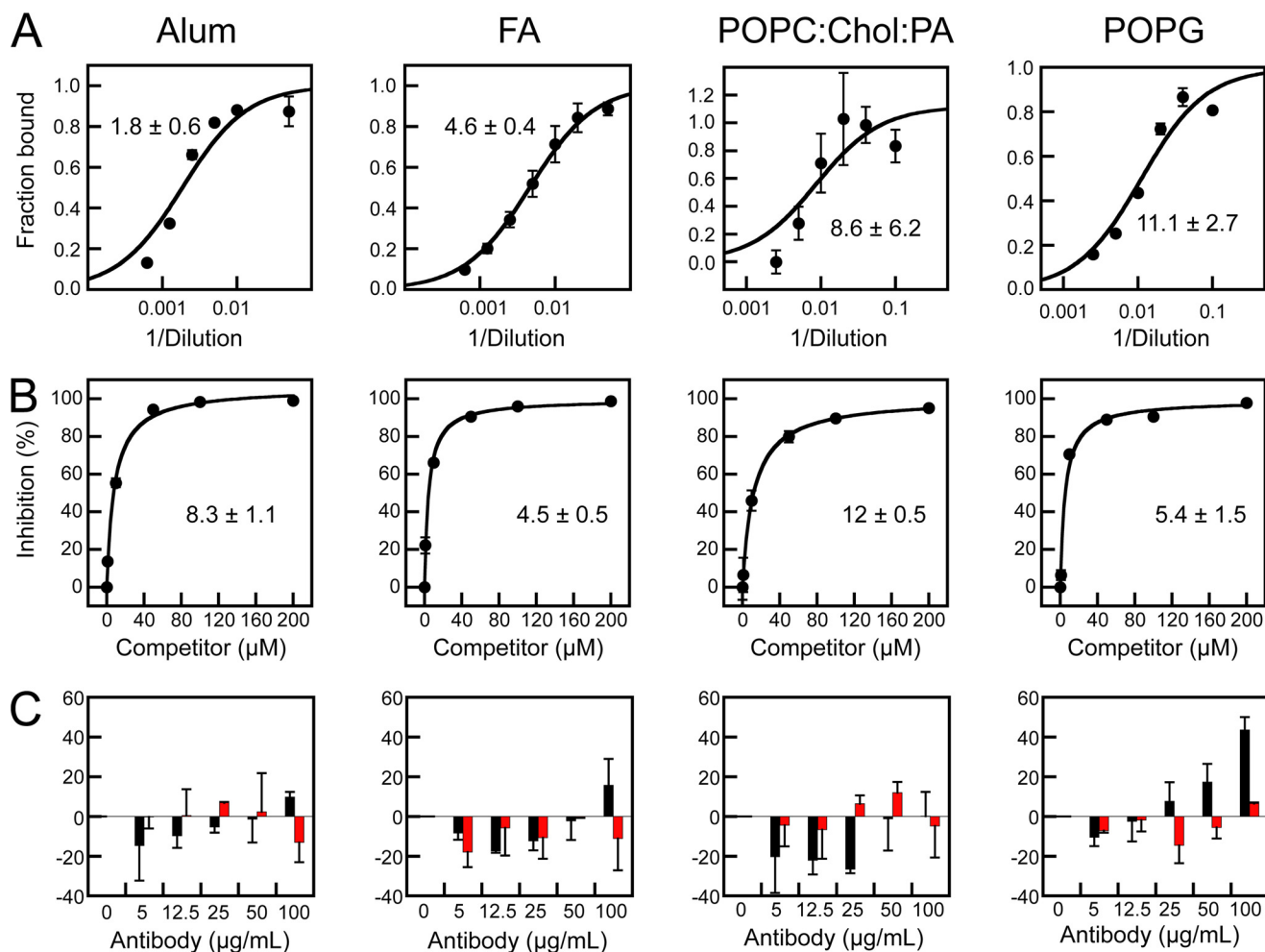


FIGURE 7. Immunogenicity of MPERp formulated with different adjuvants. *A*, midpoint IgG titers in sera from rabbits immunized with MPERp in the different adjuvants. Sera were titrated in ELISA using 1.4 μM MPERp. Experimental values were adjusted to sigmoid dose-response curves, and midpoint titers were determined as EC₅₀ values (*i.e.* the dilutions giving 50% response between minimum and maximum). Values displayed in panels correspond to 1/dilution × 10³ ± S.E. *B*, affinity for the 2F5 epitope of rabbit IgG purified from sera with 2F5ep-Cys (NEQELLELDKWASLWN-C) peptide immobilized onto a beaded agarose support. Competitive ELISAs were performed using plates coated with MPERp (1.4 μM). Prior to adding to the plates, 0.1 μg/ml of 2F5-specific IgG was preincubated for 30 min with serial dilutions of soluble 2F5ep (NEQELLELDKWASLWN) peptide. Percentages of binding inhibition were adjusted to saturation curves, which were subsequently used to infer the IC₅₀ values ± S.E. displayed in the panels. *C*, inhibition of cell entry. In these assays HIV-Env pseudoviruses were preincubated with increasing amounts of purified antibodies, and infection of TZM-bl target cells was subsequently monitored by flow cytometry as in Fig. 5A. Plotted inhibition percentage values are means of four experimental determinations. The red columns correspond to the level of neutralization exerted on VSV-G-pseudotyped viruses used as negative control.

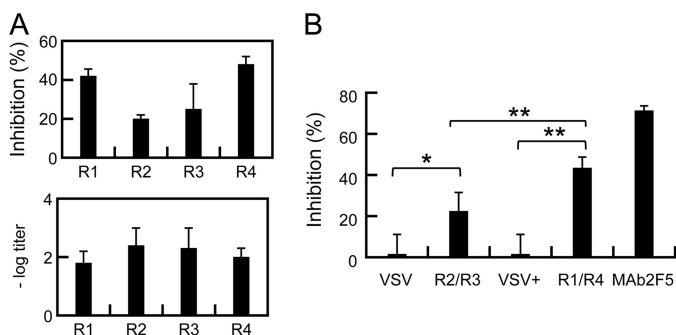


FIGURE 8. Recovered responses after vaccination with the POPG/lipid A-MPERp formulation. *A*, midpoint IgG titers (negative log EC₅₀ dilution values) and inhibition of cell entry by 2F5-specific antibodies in the sera of four different rabbits (R1–R4). Antibodies purified with 2F5ep-Cys were used at 100 μg/ml in the latter assay. *B*, levels of inhibition of cell entry mediated by HIV-1-Env or VSV-GP controls. Rabbits were classified in two groups according to significant differences in the levels of inhibition (R1/R4 versus R2/R3). Significant inhibition of Env-HIV-1 versus VSV-GP was nonetheless observed for both groups (**, $p < 0.005$; *, $p < 0.05$). MAb2F5 (2 μg/ml) was included as positive control. Means ± S.D. of six determinations are shown.

1B). Thus, the finding that these residues may also exist as part of an elongated α -helix (HFIP structure, Fig. 4A) or form a kink while retaining conformational features of an α -helix (DPC structure, Fig. 4A), underscores the structural metastability proposed for this joint sequence (73).

Unprecedented structures were additionally observed at the MPERp N-terminal stretch. The HFIP structure displayed a kink involving the ⁶⁶⁵KW⁶⁶⁶ residues, while the DPC structure displayed a ₃₁₀-helix for the ⁶⁶¹LEL⁶⁶³ residues, which also resulted in a change of the backbone direction (Fig. 4A). The presence of these structures would be consistent with the flexibility found in the case of shorter peptides (35). Moreover, they would be consistent with the type I β -turn adopted by the ⁶⁶⁴DKW⁶⁶⁶ core epitope sequence in Fab2F5-peptide complexes (14, 36, 78). In contrast, our NMR structures did not display the extended conformation found in the crystal structures of those complexes for the preceding N-terminal residues ⁶⁵⁶NEQELLE⁶⁶³ (14, 36). In both NMR structures

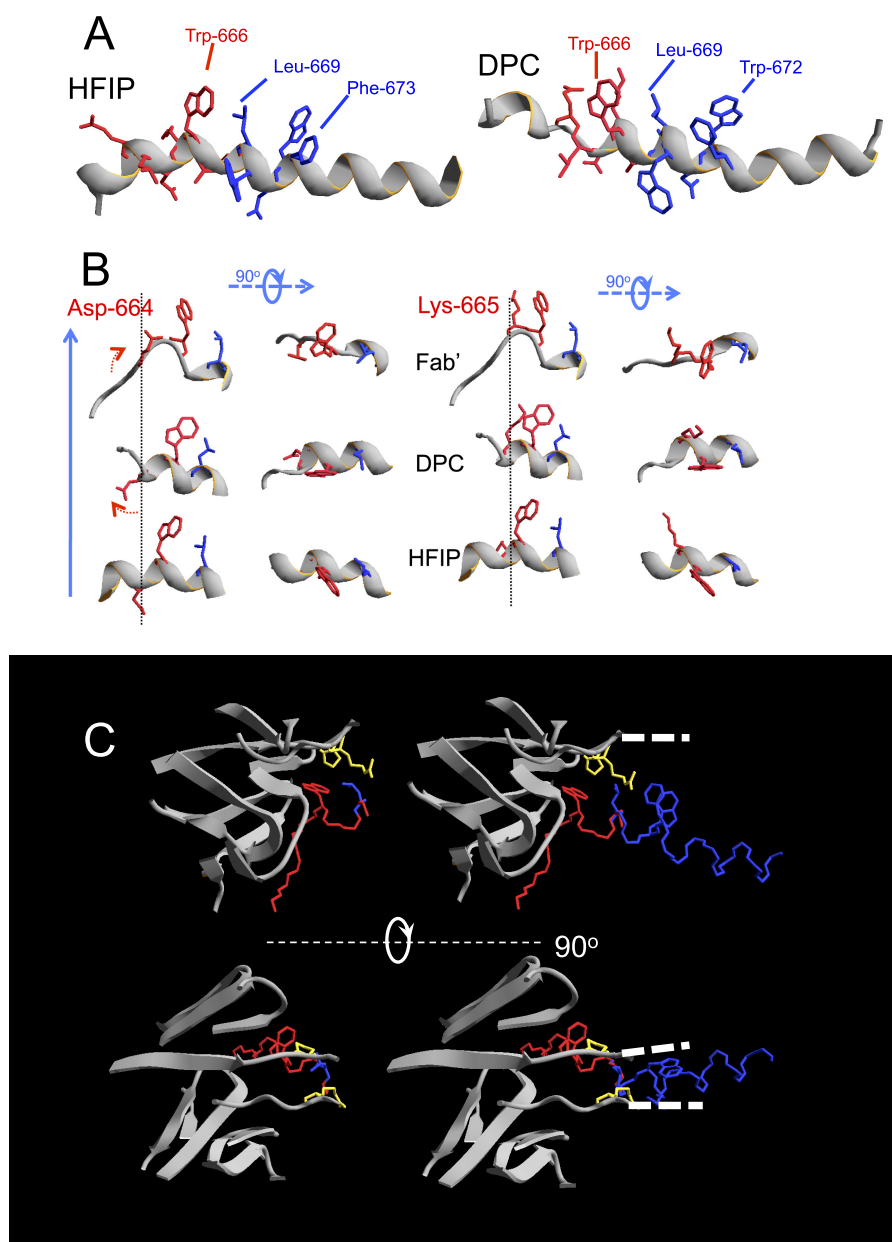


FIGURE 9. 2F5 epitope organization in MPERp and putative mechanism of antibody recognition. *A*, 2F5 epitope in HFIP and DPC structures. Core epitope residues ELDKWA are shown in red, and downstream residues putatively implied in secondary interactions with CDR-H3 loop are depicted in blue. Trp-666 and Leu-669/Trp-672/Phe-673 are displayed on the same side of the helix. *B*, comparison of 2F5 epitope structure in Fab' complex (PDB code 3D0L) and MPERp. The chain portion spanning residues Leu-661–Trp-670 is shown in gray in the three structures, with projecting side chains of Asp-664 (left) or Lys-665 (right) and Trp-666 in red. Side chain of Leu-669 is displayed in blue to establish the relative position of the downstream helix. The comparison suggests that the 3_{10} -helix observed in DPC might include an intermediate of the conformational change required for positioning Asp-664 side chain into the 2F5 paratope. Lys-665 accommodation into the paratope would not require by comparison major conformational changes of the peptide backbone. *C*, fitting of the MPERp helix into Fab'-bound peptide. The Fab paratope structure (PDB code 3D0L) is displayed in ribbon representation. The base of the flexible loop of the heavy chain (not solved in the crystal) is marked by the yellow side chains of residues Pro-98 and Arg-100B. The MPER residues Trp-666 and Leu-669 in the bound peptide are displayed in red and blue, respectively. In the right panel, the helix turn of MPERp (DPC structure) containing Leu-669 (displayed in blue) has been fitted into the Fab-bound structure. The dotted lines mark the estimated position of the loop relative to the MPERp helix.

reported here, these residues rather adopted a helical conformation (Fig. 4A).

Even though one should be cautious in interpreting a peptide's conformational states and extrapolating back to the native functional protein, the potential relevance of the helical conformation adopted by MPERp N-terminal residues is emphasized by the structure of an antigenically near-native Env construct termed "SOSIP" gp140 (79). Although truncated at position 664, the recently solved crystal structure at 4.7 Å pro-

vides insights into the gp41 ectodomain organization in the context of cleaved, stabilized HIV-1 Env trimers (80). The SOSIP structure supports the location of the 656 NEQELLE 663 residues into a solvent-exposed helix within the native Env structure.

One crystallographic structure of the 2F5 Fab complexed with peptide further displayed the turn sequence 664 DKW 666 followed by residues 667 ASLW 670 adopting a canonical α -helix conformation (36), which is also present in our NMR structures

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(Fig. 4A, see also Fig. 9C). Thus, in combination, the structural evidence suggests that the native structure recognized by the 2F5 antibody might consist of a continuous helical structure interrupted by a flexible kink at positions 664–666 that redirects the gp41 backbone at the pre-transmembrane region.

Implications for 2F5 Epitope Recognition Mechanism—The MPERp NMR structures solved in this work recreate kinked helical motifs (Fig. 9A), which, in combination with previously reported structures (Fig. 9B), sustain proposals that this region has evolved to sample alternative conformations after activation of the fusion cascade (25). Within this context, a putative mechanism for 2F5 epitope recognition is presented in Fig. 9B. The figure displays the orientations adopted by the ⁶⁶⁴DKW⁶⁶⁶ residues in MPERp structures and the Fab-bound peptide. The Trp-666 and Leu-669 side chains are oriented in parallel in the three structures, while the negative charge of Asp-664 side-chain projects from the main axis in different directions (Fig. 9B, left). By contrast, the alkyl- π stacking between Lys-665 and Trp-666 side chains found in contact with Fab could be fairly reproduced by the structure solved in the DPC structure (Fig. 9B, right). In the HFIP structure, further rotation of the Lys-665 side chain would allow its insertion into the Fab binding pocket, without requiring major changes of the peptide backbone conformation. Thus, the NMR structures suggest that binding to a helical MPER peptide might first involve contacting Lys-665, Trp-666, and Leu-669 residues and then require induction by the antibody of a conformational transition in the C _{α} chain for inserting Asp-664 into the binding pocket. Comparison of the three structures further suggests that the short _{3₁₀}-helix found in the DPC structure may encompass an intermediate between the fully helical and the extended conformations observed in HFIP- and Fab-bound structures, respectively.

The NMR structures described in this work may in addition provide insights into secondary interactions of the 2F5 antibody with MPER residues C-terminal to the core epitope (Fig. 9C). Screening of phage-displayed peptide libraries with the MAb2F5 identified Leu-669 as an almost invariant residue at the C terminus of the core epitope (63). Further competition ELISA demonstrated that the CDR-H3 loop increased binding affinity when C-terminal ⁶⁷²WFNITNWLWYIK⁶⁸³ residues were added to the full ⁶⁵⁶NEQELLELDKWASLWN⁶⁸¹ epitope sequence (38). This finding raised the possibility that the neutralization dependence on the loop apex was caused by weaker secondary binding to C-terminal MPER residues (38).

Recently reported compelling mutagenesis of the CDR-H3 loop by Güenaga and Wyatt (25) supports that idea. A significant correlation was found between neutralization potency of CDR-H3 mutants and affinity to an MPER peptide spanning residues ⁶⁵⁷EQELLELDKWASLWNWFNITNWLWYIK⁶⁸³. This correlation was lost in the case of the ⁶⁵⁹ELLELDKWASL⁶⁶⁹ sequence structurally constrained into a protein scaffold (30). Moreover, L669A, W670A, N671A, W672A, and F673A substitutions, in residues immediately C-terminal to the core epitope, resulted in an affinity decrease. It was further proposed that weak contacts involving π stacking interactions among aromatic residues present in the antibody CDR-H3 loop and the MPER peptide sequence might be responsible for this effect (25). According to these authors, this mode of recognition

would in addition enable 2F5 epitope binding when MPER organizes as a helical bundle.

The MPERp structures solved in this work, displaying the relative positions of the 2F5 core epitope and the downstream residues encompassing this secondary antibody-binding site, substantiate such a hypothesis (Fig. 9C). Fitting of the MPERp DPC helix ⁶⁶⁷ASLW⁶⁷⁰ stretch into the corresponding Fab-bound structure (36) disclosed the Leu-669 side chain at the base of the CDR-H3 loop in remarkably similar orientation and aligned the MPER helix axis with the parts of the loops visible in the crystal structure. Thus, the model that emerges from combining both structures supports the possibility that the tip of the CDR-H3 loop may establish contacts with Trp-672 and Phe-673 residues on the MPER helix (25, 38).

Implications for Immunogen Design—Overall our structural data support 2F5 docking to the surface of a kinked MPERp helix exposing the invariable residues Trp-666, Leu-669, Trp-672, and Phe-673, and the putative involvement of the CDR-H3 loop in the process. In previous studies, it was hypothesized that inclusion of residues responsible for the secondary binding process would be required to elicit 2F5-like neutralizing responses (25, 36, 38). To test the immunogenicity of an MPER helix containing all elements involved in epitope recognition by the bNAb2F5, we carried out immunization studies using MPERp as a vaccine. To establish a potential structure-immunogenicity relationship, we monitored conformational changes in the vaccine formulations. IR allows performing reliable measurements (*i.e.* resolution or sensitivity are unaffected) in turbid suspensions such as aggregated membranes and particulate emulsions. Therefore, IR was the technique of choice for confirming the preservation of MPERp helical structures in the viscous media provided by the aluminum salts, “Freund’s” water-in-oil emulsion, and membrane adjuvants (Figs. 4B and 6).

Even though MPERp dispersed in alum and Freund’s adjuvant triggered effective MPER-targeting immunoresponses, the antibodies isolated from these sera were not functional in the cell entry inhibition assays (Fig. 7). It has been reported that interactions of protein antigens with the highly charged aluminum salts may destabilize their structures (81). Consistent with this finding, MPERp in contact with alum underwent significant aggregation, as denoted by the IR absorption at 1620 cm⁻¹ (Fig. 4B, left). We speculate that MPERp unfolding/aggregation in the highly polar alum medium might result in the presentation of neutralization-irrelevant 2F5 epitope structures to the B-cell receptor. In contrast, IR demonstrated the absence of significant MPERp aggregation in Freund’s reverse micelles (Fig. 4B, right, and Table 3).

It has been argued that the water molecules confined at the interfacial region of the water-in-oil reverse micelles are non-bulk-like, resulting in a low dielectric environment at the water-oil interface, reminiscent of that found at the membrane-water interface (82). Thus, amphipathic peptides in contact with reverse micelles may attain helical structures compatible with those adopted when adsorbed to membrane surfaces. In particular, this has been demonstrated using IR for the antimicrobial peptide mastoparan X (71). Our results suggest that this is also the case for MPERp in Freund’s adjuvant and POPC/Chol/PA vesicles (Table 3). We infer that hydrophobic contacts estab-

lished by MPERp with the oil-water interface may result in limited exposure of the 2F5 epitope key residues to B-cell receptors, in a way comparable with that in contact with the surface of POPC/Chol/PA vesicles (see below).

Given its proximity to the envelope surface and the unusual concentration of aromatic residues, it has been proposed that structuring coupled to membrane partitioning may play a crucial role in determining the structure and orientation of MPER epitopes, which in turn will have an impact on immunogenicity (19, 62, 72, 83). Thus, we studied in parallel the MPERp structure and immunogenicity in two different liposomal formulations (Figs. 5 and 6). 2F5 binding to the POPG-based vaccine was CDR-H3 dependent, whereas the loop was not required for binding to the POPC/Chol/PA-based vaccine (Fig. 5). Thus, only binding to the former correlated with the antibody-neutralizing function. Sustaining different modes of antibody recognition, membrane-bound MPERp helical structures also differed substantially in these vaccines (Fig. 6). The features of the MPERp IR spectrum in POPC/Chol/PA liposomes (Fig. 6A and Table 3) resembled the flexibility and solvation degree characteristic of amphipathic helices in contact with membrane surfaces (84). Accordingly, the simulations in the POPC/Chol/PA system disclosed a membrane-bound interfacial helix (Fig. 6A). In this structure, 2F5 key epitope residues were engaged in dynamic interactions with membranes and remained mostly inaccessible for binding from solution. Similarly to Freund's, the 2F5-targeting antibodies isolated from the POPC/Chol/PA sera were not functional in cell entry assays (Fig. 7).

We surmise that the conformational flexibility of the interfacial MPERp monomers put forward by the IR and MDS studies, may sustain binding to the membrane-bound MPER epitope by MAb2F5, even in the absence of the CDR-H3 loop (Fig. 5E). Thus, the lack of functional antibodies in sera of rabbits immunized with MPERp in Freund's adjuvant and POPC/Chol/PA liposomes (Fig. 7) would be consistent with the activation of B-cell receptors that recognize alternative faces/residues within monomeric, interfacial MPER helices.

Contrasting these observations, the structure adopted by MPERp in the POPG liposomes was devoid of the more flexible conformers and denoted the existence of tightly packed, buried, and solvated helices (Fig. 6B and Table 3). MDS further revealed the possibility that a protruding bundle could insert into the lipid bilayer through the C terminus of a peptide monomer and expose to solvent 2F5 key residues from adhered helices (Fig. 6B). When used as immunogens, these POPG-bound MPERp structures gave rise to antibodies with a significant capacity of blocking cell infection (Figs. 7 and 8).

We speculate that the efficacy shown by the POPG-MPERp formulation as compared with the other tested vaccines may result from a different pattern of epitope exposure to solvent. Based on the electrostatic repulsion between N-terminal MPERp Glu carboxylates and the acidic phospholipid headgroups, we already anticipated exposure of 2F5 epitope to solvent in this formulation (61). In agreement with our expectations, the N terminus of MPERp never contacted the membrane interface in the MDS (Fig. 6B). The liposomal formulation may hypothetically contribute to immunogenicity in other ways. Following recent proposals (25), it is possible that helical bundles protruding from the

membrane surface are required to recapitulate antibodies with the capacity for binding 2F5 epitope residues, even if buried within helix-helix contacts. The fact that the 2F5 CDR-H3 was required for binding to POPG-inserted MPERp argues in favor of this possibility (Fig. 5). Alternatively, as also happens in the case detected by MDS, membrane insertion through one monomer might stabilize the out-of-register MPERp helix assembly making possible full exposure of 2F5 epitope residues to solvent in the outermost monomer. The "flagpole"-like MPER structures repeated on the surface of negatively charged membranes, might additionally embody multivalent antigens for the efficient activation of B-cell receptors. Finally, these vesicles might provide a suitable environment for generating antibodies capable of binding heterotypically to peptide and lipid (9, 31).

Although significant (Fig. 8), the inhibitory activity of these antibodies was weak, particularly when compared with that of MAb2F5 (Fig. 5A). We note that the former arise from a polyclonal response and that samples containing these antibodies are devoid of the purity level of the isolated mAb. In combination, those two factors are likely to contribute to the reduction of the specific activity of the samples tested here. We also note that to qualify the 2F5-targeting antibodies recovered from the POPG sera as neutralizing antibodies, the neutralization breadth and potency should be evaluated using referenced assays and diverse viral strains and isolates (85). In this regard, an additional study, involving larger numbers of animals and comparing different immunization strategies, is currently under way with the aim to provide evidence for neutralization according to standard methods (86).

In conclusion, results in this work suggest that structural fixation through hydrophobic interactions with the membrane interface may constrain the efficacy of liposomal vaccines targeting the 2F5 epitope. However, they present the possibility that membrane-inserted MPER bundles may embody efficient 2F5-targeting immunogens. Thus, we infer that MPER flagpoles optimized for membrane insertion and/or epitope-exposure functions might exemplify a new paradigm for future design of effective liposomal vaccines targeting the 2F5 epitope.

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Supplemental Data:

Structure and Immunogenicity of a Peptide Vaccine Including the Complete HIV-1 gp41 2F5 Epitope. Implications for Antibody Recognition Mechanism and Immunogen Design

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Table S1. ^1H chemical shifts (ppm, from DSS) for peptide MPERp in a $\text{H}_2\text{O}/\text{D}_2\text{O}$ (9:1 ratio by volume) solution containing 20 mM deuterated dodecylphosphocholine (DPC) and 2 mM HEPES buffer at pH 7.0 and 25°C. Those ^{13}C chemical shifts that could be unambiguously assigned from a 2D $^1\text{H},^{13}\text{C}$ -HSQC recorded at natural ^{13}C abundance are listed in italics. Those methylene protons (β or γ) and Leu methyl groups that were stereo-specifically assigned are indicated by an asterisk.

Residue	HN	C_αH	C_βH	Others
Asn 1		4.14	2.89, 2.89	$\text{N}_\delta\text{H}_2$ 6.99, 7.70
Glu 2		4.26	1.98, 2.08	$\text{C}_{\gamma\gamma}\text{H}$ 2.29, 2.29, 33.9
Gln 3	8.41	4.25	2.03, 2.09	$\text{C}_{\gamma\gamma}\text{H}$ 2.36, 2.36; $\text{N}_\epsilon\text{H}_2$ 6.85, 7.63
Glu 4	8.32	4.25	1.96, 2.04	$\text{C}_{\gamma\gamma}\text{H}$ 2.26, 2.26, 36.4
Leu 5	8.29	4.27	1.58, 1.71	C_γH 1.67; $\text{C}_\delta\text{H}_3$ 0.87, 23.8; $\text{C}_\delta'\text{H}_3$ 0.92, 25.1
Leu 6	8.06	4.36	1.56, 1.66	C_γH 1.61; $\text{C}_\delta\text{H}_3$ 0.83, 23.8; $\text{C}_\delta'\text{H}_3$ 0.87, 25.2
Glu 7	8.25	4.32	1.98, 2.09*	$\text{C}_{\gamma\gamma}\text{H}$ 2.23, 2.29, 36.4
Leu 8	8.29	4.18	1.61, 1.71*	C_γH 1.71; $\text{C}_\delta\text{H}_3$ 0.84, 23.8; $\text{C}_\delta'\text{H}_3$ 0.92, 25.1
Asp 9	8.48	4.50	2.72, 2.72	
Lys 10	8.08	4.08	1.72, 1.63*	$\text{C}_{\gamma\gamma}\text{H}$ 1.16, 1.16; $\text{C}_{\delta\delta}\text{H}$ 1.51, 1.51; $\text{C}_{\epsilon\epsilon}\text{H}$ 2.80, 2.80, 41.6
Trp 11	7.94	4.53	3.43, 3.24*	$\text{C}_{\delta 1}\text{H}$ 7.38; $\text{N}_{\epsilon 1}\text{H}$ 10.65; $\text{C}_{\epsilon 3}\text{H}$ 7.51; $\text{C}_{\zeta 3}\text{H}$ 6.92; $\text{C}_{\eta 2}\text{H}$ 7.06; $\text{C}_{\zeta 2}\text{H}$ 7.42
Ala 12	8.11	4.08	1.51, 1.85	
Ser 13	8.10	4.31	3.90, 3.98	
Leu 14	7.83	4.15	1.79, 1.65*	C_γH 1.66; $\text{C}_\delta\text{H}_3$ 0.79*; $\text{C}_\delta'\text{H}_3$ 0.85*
Trp 15	7.94	4.59	3.43, 3.24*	$\text{C}_{\delta 1}\text{H}$ 7.25; $\text{N}_{\epsilon 1}\text{H}$ 10.30; $\text{C}_{\epsilon 3}\text{H}$ 7.54; $\text{C}_{\zeta 3}\text{H}$ 6.91; $\text{C}_{\eta 2}\text{H}$ 7.04; $\text{C}_{\zeta 2}\text{H}$ 7.41
Asn 16	8.02	4.58	2.82, 2.88	$\text{N}_\delta\text{H}_2$ 7.00, 7.63
Trp 17	nd	4.37	3.31, 3.31	$\text{C}_{\delta 1}\text{H}$ 7.18; $\text{N}_{\epsilon 1}\text{H}$ 10.50; $\text{C}_{\epsilon 3}\text{H}$ 7.36; $\text{C}_{\zeta 3}\text{H}$ 6.91; $\text{C}_{\eta 2}\text{H}$ 7.06; $\text{C}_{\zeta 2}\text{H}$ 7.44
Phe 18	7.98	4.22	2.95, 3.10*	$\text{C}_{\delta\delta}\text{H}$ 7.21, 7.21; $\text{C}_{\epsilon\epsilon}\text{H}$ 7.12, 7.12; C_ζH nd
Asn 19	7.64	4.55	2.62, 2.81	$\text{N}_\delta\text{H}_2$ 7.67, 6.70*

Ile 20	8.12	3.67	1.61	$C_{\gamma\gamma}H$ 1.14, 1.42*; $C_{\gamma}H_3$ 0.76, 17.8; $C_{\delta}H_3$ 0.75, 13.5
Thr 21	8.03	3.85	4.02	$C_{\gamma}H_3$ 1.05, 21.9
Asn 22	7.90	4.57	2.69, 2.69	$N_{\delta}H_2$ 6.70, 7.59
Trp 23	7.99	4.58	3.33, 3.33	$C_{\delta_1}H$ 7.19; $N_{\epsilon_1}H$ 10.27; $C_{\epsilon_3}H$ 7.42; $C_{\zeta_3}H$ 6.85; $C_{\eta_2}H$ 6.98; $C_{\zeta_2}H$ 7.33
Leu 24	8.23	4.03	1.50, 1.79*	$C_{\gamma}H$ 1.79; $C_{\delta}H_3$ 0.91*; $C_{\delta'}H_3$ 0.85*
Trp 25	7.93	4.26	3.33, 3.33	$C_{\delta_1}H$ 7.16; $N_{\epsilon_1}H$ 10.24; $C_{\epsilon_3}H$ 7.45; $C_{\zeta_3}H$ 7.06; $C_{\eta_2}H$ 7.21; $C_{\zeta_2}H$ 7.49
Tyr 26	7.74	4.00	3.00, 3.12	$C_{\delta\delta'}H$ 7.09, 7.09; $C_{\epsilon\epsilon'}H$ 6.81, 6.81
Ile 27	7.83	3.77	1.92	$C_{\gamma\gamma}H$ 1.64, 1.30*; $C_{\gamma}H_3$ 0.90, 18.2; $C_{\delta}H_3$ 0.88, 13.7
Lys 28	7.69	4.04	1.73, 1.80	$C_{\gamma\gamma}H$ 1.41, 1.47; $C_{\delta\delta'}H$ 1.58, 1.58; $C_{\epsilon\epsilon'}H$ 2.81, 2.81, 41.6
<i>CONH₂</i>	6.96, 7.26			

Table S2. ^1H chemical shifts (ppm, from DSS) for peptide MPERp in a $\text{H}_2\text{O}/\text{D}_2\text{O}$ (9:1 ratio by volume) solution containing 2 mM HEPES buffer at pH 7.0, and 25 % HFIP at 25°C. Those ^{13}C chemical shifts that could be unambiguously assigned from a 2D ^1H , ^{13}C -HSQC recorded at natural ^{13}C abundance are listed in italics. An asterisk indicates the stereo-specifically assigned methylene protons. nd stands for not determined.

Residue	HN	C_αH	C_βH	Others
Asn 1		nd	3.21, 3.36, <i>37.0</i>	$\text{N}_\delta\text{H}_2$ 6.99, 7.72
Glu 2		4.17	2.15, 2.15	$\text{C}_{\gamma\gamma}\text{H}$ 2.49, 2.49, <i>33.9</i>
Gln 3	8.34	4.04	2.15, 2.18	$\text{C}_{\gamma\gamma}\text{H}$ 2.46, 2.46, <i>33.9</i> ; $\text{N}_\epsilon\text{H}_2$ nd
Glu 4	8.09	4.09	2.13, 2.20	$\text{C}_{\gamma\gamma}\text{H}$ 2.45, 2.52, <i>33.7</i>
Leu 5	7.56	4.22	1.77, 1.83	C_γH 1.76; $\text{C}_\delta\text{H}_3$ 0.93, <i>23.1</i> ; $\text{C}_\delta'\text{H}_3$ 0.99, <i>23.7</i>
Leu 6	7.91	4.18	1.65, 1.93	C_γH 1.83; $\text{C}_\delta\text{H}_3$ 0.84, <i>23.5</i> ; $\text{C}_\delta'\text{H}_3$ 0.97, <i>24.3</i>
Glu 7	7.92	4.17	2.21, 2.27	$\text{C}_{\gamma\gamma}\text{H}$ 2.49, 2.58, <i>34.4</i>
Leu 8	8.17	4.30	1.88, 1.88	C_γH 1.83; $\text{C}_\delta\text{H}_3$ 0.94, <i>22.5</i> ; $\text{C}_\delta'\text{H}_3$ 1.00, <i>23.5</i>
Asp 9	8.48	4.50	2.92, 3.01	
Lys 10	8.17	4.09	2.00, 2.07	$\text{C}_{\gamma\gamma}\text{H}$ 1.42, 1.60; $\text{C}_{\delta\delta}\text{H}$ 1.68, 1.68; $\text{C}_{\epsilon\epsilon}\text{H}$ 2.88, 2.88, <i>42.1</i>
Trp 11	8.33	4.48	3.40, 3.51	$\text{C}_{\delta 1}\text{H}$ 7.11; $\text{N}_{\epsilon 1}\text{H}$ 9.06; $\text{C}_{\epsilon 3}\text{H}$ 7.64; $\text{C}_{\zeta 3}\text{H}$ 7.04; $\text{C}_{\eta 2}\text{H}$ 6.97; $\text{C}_{\zeta 2}\text{H}$ 7.11
Ala 12	9.12	3.98	1.61, <i>17.6</i>	
Ser 13	8.15	4.32	nd	
Leu 14	7.99	4.29	1.73, 1.83	C_γH 1.71; $\text{C}_\delta\text{H}_3$ 0.88, <i>23.4</i> ; $\text{C}_\delta'\text{H}_3$ 0.92, <i>23.8</i>
Trp 15	8.61	4.39	2.80, 3.13	$\text{C}_{\delta 1}\text{H}$ 7.25; $\text{N}_{\epsilon 1}\text{H}$ 8.96; $\text{C}_{\epsilon 3}\text{H}$ 7.52; $\text{C}_{\zeta 3}\text{H}$ 7.06; $\text{C}_{\eta 2}\text{H}$ 7.24; $\text{C}_{\zeta 2}\text{H}$ 7.44
Asn 16	8.46	4.42	2.77, 3.08	$\text{N}_\delta\text{H}_2$ 6.34, 7.37
Trp 17	8.30	4.36	3.42, 3.59*	$\text{C}_{\delta 1}\text{H}$ 7.01; $\text{N}_{\epsilon 1}\text{H}$ 9.02; $\text{C}_{\epsilon 3}\text{H}$ 7.57; $\text{C}_{\zeta 3}\text{H}$ 7.06; $\text{C}_{\eta 2}\text{H}$ 7.18; $\text{C}_{\zeta 2}\text{H}$ 7.36
Phe 18	9.14	3.84	3.12, 3.21	$\text{C}_{\delta\delta}\text{H}$ 7.09, 7.09; $\text{C}_{\epsilon\epsilon}\text{H}$ 7.29, 7.29; C_ζH nd
Asn 19	7.98	4.20	2.39, 2.66	$\text{N}_\delta\text{H}_2$ nd

Ile 20	8.45	3.83	1.82	$C_{\gamma\gamma}H$ 1.14, 1.70; $C_{\gamma}H_3$ 0.93, 16.6; $C_{\delta}H_3$ 0.85, 12.4
Thr 21	7.98	3.73	3.71	$C_{\gamma}H_3$ 0.70, 20.4
Asn 22	7.62	4.44	2.47, 2.65	$N_{\delta}H_2$ 5.32, 6.73
Trp 23	7.99		3.34, 3.53	$C_{\delta_1}H$ 7.09; $N_{\epsilon_1}H$ 9.09; $C_{\epsilon_3}H$ 7.57; $C_{\zeta_3}H$ 7.01; $C_{\eta_2}H$ 7.17; $C_{\zeta_2}H$ 7.36
Leu 24	8.46	3.87	1.84, 1.40*	$C_{\gamma}H$ 1.85; $C_{\delta}H_3$ 0.84, 22.4; $C_{\delta'}H_3$ 0.86, 24.4
Trp 25	8.11	4.20	3.27, 3.32	$C_{\delta_1}H$ 6.96; $N_{\epsilon_1}H$ 9.41; $C_{\epsilon_3}H$ 7.49; $C_{\zeta_3}H$ 7.11; $C_{\eta_2}H$ 7.22; $C_{\zeta_2}H$ 7.42
Tyr 26	7.83	4.09	3.00, 3.11	$C_{\delta\delta'}H$ 7.06, 7.06; $C_{\epsilon\epsilon'}H$ 6.85, 6.85
Ile 27	8.11	3.73	1.70	$C_{\gamma\gamma}H$ 1.10, 1.23; $C_{\gamma}H_3$ 0.66, 17.0; $C_{\delta}H_3$ 0.65, 11.8
Lys 28	8.00	4.03	1.78, 1.78	$C_{\gamma\gamma}H$ 1.39, 1.39; $C_{\delta\delta'}H$ 1.58, 1.58; $C_{\epsilon\epsilon'}H$ 2.84, 2.84, 41.8
<i>CONH₂</i>	6.66, 6.92			

Structure and Immunogenicity of a Peptide Vaccine, Including the Complete HIV-1 gp41 2F5 Epitope: IMPLICATIONS FOR ANTIBODY RECOGNITION MECHANISM AND IMMUNOGEN DESIGN

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