THE BROADLY NEUTRALIZING ANTI-HIV-1 4E10 MONOCLONAL ANTIBODY IS BETTER ADAPTED TO MEMBRANE-BOUND EPITOPE RECOGNITION AND BLOCKING THAN 2F5

Nerea Huarte¹, Maier Lorizate¹*, Rubén Maeso¹, Renate Kunert², Rocio Arranz³, José M. Valpuesta³ and José L. Nieva¹

¹Biophysics Unit (CSIC-UPV/EHU) and Biochemistry and Molecular Biology Department, University of the Basque Country, PO Box 644, 48080 Bilbao, Spain.

²Institute of Applied Microbiology, University of Agriculture, A-1190 Vienna, Austria.

³Department of Macromolecular Structures, National Center for Biotechnology (CSIC), Cantoblanco, 28049 Madrid, Spain.

Running title: NAb blocking of membrane-active HIV-1 sequences

*Present Address: Abteilung Virologie, Universitätsklinikum Heidelberg, im Neuenheimer Feld 324, D-69120 Heidelberg, Germany.

To whom enquiries should be addressed: José L. Nieva. Unidad de Biofísica (CSIC-UPV/EHU), Universidad del País Vasco, Aptdo 644, 48080 Bilbao, Spain. Phone: 34 94 6013353/ Fax: 34 94 6013360 E-mail: gbpnesj@lg.ehu.es.

Words in abstract: 233

Words in text: 5705
Abstract:

The broadly neutralizing 2F5 and 4E10 monoclonal antibodies (MAbs) recognize epitopes within the membrane proximal external region (MPER) that connects the HIV-1 envelope gp41 ectodomain with the transmembrane anchor. By adopting different conformations that stably insert into the virion external membrane-interface, such as helical structures, a conserved aromatic-rich sequence within the MPER is thought to participate in HIV-1 cell fusion. Recent experimental evidence suggests that the neutralizing activity of 2F5 and 4E10 might correlate with the MAbs capacity to recognize epitopes inserted into the viral membrane, thereby impairing MPER fusogenic activity. To gain new insights into the molecular mechanism underlying viral neutralization by these antibodies, we have compared the capacity of 2F5 and 4E10 to block the membrane-disorganizing activity of MPER peptides inserted into the surface bilayer of solution-diffusing unilamellar vesicles. Both MAbs inhibited leakage of vesicular aqueous contents (membrane permeabilization) and inter-vesicular lipid-mixing (membrane fusion) promoted by MPER-derived peptides. Thus, our data support that antibody binding to a membrane-inserted epitope may interfere with the function of the MPER during gp41-induced fusion. Antibody insertion into a cholesterol-containing, uncharged virion-like membrane is mediated by specific epitope recognition and moreover, partitioning-coupled folding into a helix reduces the efficiency of the 2F5 Mab binding to its epitope in the membrane. We conclude that the capacity to interfere with the membrane activity of conserved MPER sequences is best correlated with the broad neutralization of the 4E10 MAb.
**Introduction**

It is generally accepted that a potentially successful HIV vaccine should be capable of eliciting a robust neutralizing antibody response (15, 16). The isolation from infected asymptomatic individuals of several antibodies showing neutralizing activity against a broad spectrum of viral clades implies that triggering such immunogenic response might be conceivably possible (15, 16, 74). The anti-gp41 2F5 and 4E10 monoclonal antibodies (MAbs) constitute a paradigm in this respect (21, 34, 48, 49, 56, 68). These MAbs simultaneously exert broad and potent neutralizing activity across different HIV-1 strains and primary isolates (9, 34, 45, 48, 68, 89), confer protection to viral infection when passively transferred to primate models (67) and increase the neutralization response upon passive immunization of humans (66, 74). Thus, deciphering the molecular mechanisms underlying viral cross-neutralization by 2F5 and 4E10 poses a challenge for researchers working on HIV vaccine development (86).

2F5 and 4E10 recognize conserved linear epitopes located within the membrane-proximal external region (MPER) of the HIV-1 gp41 fusogenic Env subunit (7, 18, 19, 34, 48, 49, 51, 54, 89). The crystal structures of the 2F5 and 4E10 Fabs have been resolved in a complex with soluble epitope derivatives (13, 18, 19, 51), revealing long CDR H3s containing solvent-exposed aromatic residues. Aromatic residues are the most hydrophobic-at-interface residues and as such, they are postulated to efficiently drive water-membrane partitioning (81, 83), suggesting that these antibodies share a common adaptive trait for epitope recognition at the membrane surface (19, 51, 62). Hydropathy plots calculated according to the Wimley-White (WW) scale (81) actually revealed a fully hydrophobic-at-
interface sequence within gp41 MPER, previously termed the pre-transmembrane domain (PreTM) (39, 50, 59, 70, 71). Thus the gp41 region spanning 2F5 and 4E10 epitopes has a specific affinity for the membrane interface, the lipid bilayer region that intervenes the high-polarity water phase and the low-polarity hydrocarbon core (79).

Subsequent development of WW scale applications, including the simultaneous computation of interfacial affinity and hydrophobic moments (38, 58), revealed that the MPER was segmented into two subdomains, a phenomenon that was proposed to be functionally meaningful (58). These analyses predicted the existence of a helical, conserved amphipathic-at-interface sequence (AIS), located at the amino-terminal end of PreTM, which would be followed by a fully hydrophobic-at-interface stretch (Figure 1). The Fab structures solved in complex with epitope-representing peptides revealed the sequences in contact with 2F5 (657EQELLELDKWASLW670) and 4E10 paratopes (672WFNITNWLW680) (18, 51). Thus, the full 2F5 epitope roughly corresponds to the AIS, while the 4E10 epitope starts at the junction between the subdomains and ends close to the PreTM C-terminus (Figure 1A).

Recent structural characterization by Sun et al. (72) combining nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR) spectroscopy supported this predicted membrane topology. Indeed, these data confirmed that a MPER-derived peptide adopts a bipartite membrane structure, consisting of a tilted amphipathic N-terminal helix (residues 664-672) connected via a short hinge to a C-terminal helix (residues 675-683) mostly embedded in the membrane interface (Figure 1B). The peptide analyzed by these authors spanned residues 662-683, slightly longer than the PreTM (residues 664-683), and it did not include the complete AIS sequence (residues 656-671, Figure 1A).
Mutagenesis studies and biophysical characterization of representative peptides further suggest an active role for MPER in gp41-mediated fusion (24, 25, 39, 47, 59, 60, 64, 65, 70-72, 76), either by transmitting protein conformational energy into the virion membrane, and/or by perturbing its integrity (Figure 1B-left). Cryoelectron tomography analysis of intact SIV virions supports the notion that membrane-inserted MPER sequences may indeed represent relevant native gp41 structures (57, 85). Thus, the neutralization efficiency of the 2F5 and 4E10 MAbs may conceivably be dependent on their capacity to bind membrane-inserted MPER sequences and subsequently interfere with their membrane-disorganizing activity during the fusion reaction cycle (Figure 1B-right). Here, we tested this hypothesis by comparing the in-membrane recognition and blocking activity of the 2F5 and 4E10 MAbs. We have used solution-diffusing, unstressed phospholipid vesicles with sizes that approximate to that of the HIV virion (i.e., with a comparable bilayer intrinsic curvature), and an MPER-derived sequence (AISpreTM) that combines the full length 2F5 and 4E10 epitopes (13, 18, 51, 54). The apparent in-membrane MAb-epitope binding parameters were inferred from the inhibition of peptide-induced leakage and fusion in this intact system (i.e., without separating or diluting bound and unbound species). Together with direct electron microscopy and flow cytometry determination of the vesicle-antibody association, the inhibition data defined a comparatively lower affinity of the 2F5 MAb for membrane-bound species.
**Materials and Methods**

*Materials:*

The sequences AISpre™, AISpre™(9, 10)A, AISpre™(17, 18), AIS, and Pre™ displayed in Figure 1A were synthesized as C-terminal carboxamides by solid-phase synthesis using Fmoc chemistry, and they were purified by HPLC at the Proteomics Unit of the University Pompeu-Fabra (Barcelona, Spain). Peptide stock solutions were prepared in dimethylsulfoxide (DMSO, spectroscopy grade) and the concentrations were determined using a Bicinchoninic Acid microassay (Pierce, Rockford, IL, USA). Neutralizing antibody expressing hybridomas were originally generated by combined PEG-electrofusion of peripheral blood mononuclear cells of HIV infected non-symptomatic patients (14). The 2F5 and 4E10 MAbs used in this study were subsequently produced in recombinant CHO cells after the subclass switch to IgG1 (33). 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and Cholesterol (Chol) were purchased from Avanti Polar Lipids (Birmingham, AL, USA), while Dodecylphosphocholine (DPC) was from Anatrace (Maumee, OH, USA). The N-(7-nitro-benz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (N-NBD-PE), N-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine (N-Rh-PE), N-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (f-DHPE), N-(5-dimethylaminonaphtalene-1-sulfonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (d-DHPE), 8-aminonaphtalene-1,3,6-trisulfonic acid sodium salt (ANTS) and p-xylenebis(pyridinium)bromide (DPX) fluorescent probes were from...
Molecular Probes (Junction City, OR, USA), while the PE-Cy5 mouse anti-human IgG was purchased from BD Biosciences.

Infrared spectroscopy

Infrared spectra were recorded at 25 ºC in a Bruker Tensor 27 spectrometer equipped with a mercury-cadmium-telluride detector using a peltier based temperature controller (TempCon, BioTools Inc., Wauconda, IL) with calcium fluoride cells (BioCell, BioTools Inc., Wauconda, IL). Typically, 370 scans were collected for each background and peptide-lipid sample prepared in D$_2$O-based buffer, 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 in detail elsewhere (4-6).

Production of vesicles

Large unilamellar vesicles (LUV) were prepared following the extrusion method of Hope et al. (29). Phospholipids and cholesterol were mixed in chloroform and dried under a N$_2$ stream. Traces of organic solvent were removed by overnight vacuum pumping. Subsequently, the dried lipid films were dispersed in 5 mM Hepes, 100 mM NaCl (pH 7.4) buffer, and subjected to 10 freeze-thaw cycles prior to extrusion 10 times through 2 stacked polycarbonate membranes (Nuclepore, Inc., Pleasanton, CA, USA). The size distributions of the vesicles were determined using a Malvern Zeta-Sizer Nano ZS instrument (Malvern Instruments, Malvern, UK). Extrusion through membranes with a nominal pore-size of 0.1 and 0.2 µm produced POPC:Chol (2:1 mol:mol) LUV with mean diameters (± S.D.) of
90±17 and 132±36 nm respectively. Lipid concentrations of liposome suspensions were determined by phosphate analysis.

**Fluorimetric assays**

Peptide partitioning into membranes was evaluated by monitoring the change in the emitted Trp-fluorescence. Corrected spectra were recorded using a SLM Aminco 8100 spectrofluorimeter (Spectronic Instruments, Rochester, NY) with excitation set at 280 nm and 5-nm slits. Partitioning curves were subsequently computed from the fractional changes in emitted Trp-fluorescence when titrated with increasing lipid concentrations. The signal was further corrected for dilution and inner filter effects (80) using NATA, a soluble Trp analogue that does not partition into the membranes. The apparent mole fraction partition coefficients, $K_x$, were determined by fitting the experimental values to a hyperbolic function:

$$\frac{F}{F_0} = 1 + \frac{[(F_{\text{max}}/F_0)-1][L]}{K+[L]} \quad [1]$$

Where $F$ and $F_0$, are the fluorescence intensities measured in the presence and absence of vesicles, respectively, [L] is the lipid concentration and K is the lipid concentration at which the bound peptide fraction is 0.5. Therefore, $K_x = [W]/K$ where [W] is the molar concentration of water. Experimental free energies of partitioning (Table 1) were subsequently inferred from the following expression (79):

$$\Delta G_{\text{obs}} = -RT \ln K_x \quad [2]$$
The release of vesicular contents into the medium was monitored by the ANTS/DPX assay (23). LUV containing 12.5 mM ANTS, 45 mM DPX, 20 mM NaCl and 5 mM Hepes were obtained by separating the unencapsulated material by gel filtration on a Sephadex G-75 column eluted with 5 mM Hepes, 100 mM NaCl (pH 7.4). Osmolarities were adjusted to 200 mosm in a cryoscopic osmometer (Osmomat 030, Gonotec, Berlin, Germany). Fluorescence measurements were performed by setting ANTS emission at 520 nm and excitation at 355 nm. A cutoff filter (470 nm) was placed between the sample and the emission monochromator. The 0% leakage corresponded to the fluorescence of the vesicles at time zero whereas 100% leakage was the fluorescence value obtained after addition of Triton X-100 (0.5%, v/v).

Partitioning into the membrane interface was measured as a function of time by energy transfer from the Trp peptide to the surface d-DHPE fluorescent probe as in (37). In brief, 6 mol % of the d-DHPE probe was included in the target vesicle composition and its fluorescence was measured at an emission wavelength of 510 nm, while the excitation wavelength was that of the Trp residue (280 nm).

Membrane lipid mixing was monitored using the resonance energy transfer (RET) assay described by Struck et al. (69). The assay is based on the dilution of N-NBD-PE and N-Rh-PE, whereby dilution due to membrane mixing results in increased N-NBD-PE fluorescence. Vesicles containing 0.6 mol % of each probe were mixed with unlabeled vesicles at a ratio of 1:4 (final lipid concentration, 0.1 mM). The NBD emission was monitored at 530 nm with the excitation wavelength set at 465 nm. A cutoff filter at 515 nm was used between the sample and the emission monochromator to avoid scattering interference. The fluorescence scale was calibrated such that the zero level corresponded to
the initial residual fluorescence of the labeled vesicles and the value of 100 % corresponded to the complete mixing of all the lipids in the system. The latter value was set by the fluorescence intensity of vesicles labeled with 0.12 mol % of each fluorophore at the same total lipid concentration as in the fusion assay.

**Monolayer penetration**

Surface pressure was determined in a fixed-area circular trough (µTrough S system, Kibron, Helsinki), and the measurements were taken at room temperature and with constant stirring in an aqueous phase consisting of 1 ml 5 mM Hepes, 100 mM NaCl (pH 7.4). Lipid mixtures dissolved in chloroform were spread over the surface and the desired initial surface pressure (π₀) was attained by changing the amount of lipid applied to the air-water interface. Peptide was injected into the subphase with a Hamilton microsyringe. At the concentrations used, the peptide alone only induced a negligible increase in surface pressure at the air-water interface.

**Enzyme-linked immunosorbent assay (ELISA)**

Peptides were dissolved in phosphate-buffered saline (PBS) and immobilized (100 µl/well) overnight in C96 Maxisorp microplate wells (Nunc, Denmark) at a concentration of 1 µM. Prior to incubation with the MAbs, the plates were blocked for 2 h with 3 % (w/v) bovine-serum albumin in PBS. The binding of the MAbs was detected with an alkaline phosphatase-conjugated goat anti-human immunoglobulin (Pierce, Rockford, IL, USA),
which then catalyzed a color reaction with the p-nitrophenyl phosphate substrate (Sigma, St. Louis, MO, USA) that could be measured by absorbance at a wavelength of 405 nm in a Synergy HT microplate reader (Bio-TEK Instruments Inc., VT, USA).

Electron microscopy

Experimental evidence for stable antibody-vesicle association was initially obtained by electron microscopy. Liposome-MAb samples were prepared following a protocol analogous to that used for the cytometric analysis (see below). Samples were subsequently incubated on copper grids for 2 minutes, dried and finally stained for 1 minute with 2% uranyl acetate. Electron micrographs were obtained using a JEOL 1200EX II microscope operated at 100KV and recorded on Kodak SO-163 film.

Flow cytometry

Antibody-vesicle association was also determined quantitatively using a BD FACScalibur Flow Cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Measuring conditions were initially optimized attending to the following experimental parameters: 1) vesicle size: large unilamellar vesicles (LUVs) extruded through polycarbonate filters with nominal sizes of 100, 200, 400 and 1000 nm, and multilayered vesicles (MLVs) were compared; 2) Lipid concentration in the samples; and 3) percentage of fluorescent probe in the membrane testing 1, 0.1, 0.01 and 0.001 mol of f-DHPE. The optimal conditions were set for LUVs obtained through extrusion using filters
with a nominal pore-size of 200 nm, a lipid concentration of 0.25 mM, and 0.01 % fluorescent phospholipid probe (mole percent). Thus, in a typical experiment fluorescently labeled vesicles doped with peptide epitopes (1:100 peptide:lipid molar ratio) were incubated for 10 minutes with increasing concentrations of the 2F5 or 4E10 MAbs and subsequently, for 5 minutes with twice the concentration of anti-human immunoglobulin coupled to cyanine (IgG-PE-Cy5).

Results

Partitioning of AISpreTM into membranes: structure and energetics

The aim of the present study was to compare the ability of 2F5 and 4E10 MAbs to recognize complete membrane-inserted epitopes. Thus, the AISpreTM boundaries (residues 656-683) were defined as containing the full-length 2F5 and 4E10 epitopes, as well as both interfacial subdomains (Figure 1A). The intrinsic capacity of the designed AISpreTM sequence to translocate into membranes and fold therein was first analyzed based on its amino acid composition (Table 1). Water-to-membrane interface transfer free energies for each amino acid were determined by Wimley and White (79, 81). These values reflect real thermodynamic measurements compiling the energetic components that dictate initial partitioning of unfolded peptide sequences into membranes. Furthermore, they are additive, which allows computing the energetics of partitioning for arbitrary protein sequences. Computation of the overall free energies of partitioning for the unfolded AISpreTM and
PreTM sequences (ΔG_{nsw}-s) rendered values in the order of -2.8 and -8.1 Kcal/mol, respectively (Table 1). Following the equation 2 (Materials and Methods), these values corresponded to the partitioning constants (K_x) in the order of 10^2 and 10^6, which reflects the much higher solubility or reduced membrane affinity of the AISpreTM sequence in comparison to that of PreTM. Those K_x values actually predicted that AISpreTM partitioning would be insignificant at lipid concentrations that would otherwise permit substantial PreTM-membrane association (e.g., more than 60 % of the added peptide at a lipid concentration of 100 µM).

However, C_α chain polarity may be reduced upon folding as an α-helix, which results in an increase of the interfacial hydrophobicity (35, 78). Free energies of partitioning recalculated taking into account this conformational energy (ΔG_{nsw,s}) rendered K_x values higher than 10^7 in both cases, consistent with the quantitative incorporation of PreTM and AISpreTM into membranes. Thus, from the sequence it can be inferred that decreasing the C_α chain polarity by adopting a defined secondary structure is a prerequisite for the efficient incorporation of the AISpreTM into vesicles (35, 78, 79). In contrast, the free energy values computed for the AIS sequence predicted that this gp41 stretch alone would not associate to the membrane interface even if it adopted an α-helix structure (Table 1). This implies that the embedding of the complete 2F5 epitope into the membrane interface would require its folding into an α-helix, as well as extending the sequence at its C-terminus to include the aromatic PreTM residues.

Thus, we next set out to assess experimentally the structure adopted by AISpreTM in a membrane environment and to explore the conditions that permit the efficient incorporation
of the peptide from water into lipid bilayers (supplemental Figures S1 and S2, and supplemental Table S1). Quantitative IR determinations were indeed consistent with the helical structuring of the 2F5 and 4E10 epitopes in the context of membrane-bound AISpreTM. Decomposition of the amide I bands of PreTM and AISpreTM confirmed that these peptides adopted a very similar secondary structure in DPC micelles (Figure S1 and Table S1). In both cases the α-helical conformation was predominant, although its contribution was slightly higher in the AISpreTM sequence. By contrast, the AIS peptide amide I band could not be fitted to a major component, consistent with the absence of a single main secondary structure. In this case, a strong contribution of unordered structures co-existed with a mixture of canonical structures. Notably, bands that could be assigned to β-turns and/or 3_10-helices contributed to the same extent as α-helices and β-sheet bands. Thus, these IR results confirmed that the conformational plasticity previously reported for peptides representing the extended 2F5 epitope (7, 10) was maintained in the nonpolar environment provided by DPC micelles. Moreover, they also suggested that this flexible sequence was constrained into a main α-helical structure when anchored to the membrane interface as a part of AISpreTM.

We next analyzed experimentally AIS and AISpreTM membrane association (Figure S2). The monolayer insertion results reflected exclusion pressures (levels of lateral compression precluding insertion) of 43 and 28 mN m\(^{-1}\) for AISpreTM and AIS, respectively, indicating that AISpreTM was capable of penetrating into comparatively more packed lipid bilayers (Figure S2-A). Moreover, while AISpreTM and PreTM clearly inserted above the lateral pressures existing in biological membranes (\(\pi_0 \geq 30-35\) mN m\(^{-1}\), Table 1), it was predicted that AIS was devoid of the capacity for inserting into lipid
bilayers that are not expanded artificially (40). These observations were further confirmed
by measurements of peptide insertion into large unilamellar vesicles (LUV) (Figure S2-B).
LUV lipid bilayers are compressed to lateral pressures in the range of those existing in
natural membranes and, therefore, peptides showing exclusion pressures below 30 mN m\(^{-1}\)
are predicted not to insert into these vesicles. AISpreTM insertion into LUV bilayers could
be deduced from the changes in the fluorescence Trp spectrum obtained upon titrating
peptide with lipid vesicles (Figure S2-B). The polarity of the environment surrounding Trp
residues decreased upon penetration of the peptide into the bilayer, which resulted in Trp
fluorescence intensity enhancement and shifting of its maximum towards lower
wavelengths in the presence of the vesicles (left panel in Figure S2-B). The corresponding
partitioning curve reflects the fraction of peptide bound to vesicles as the amount of lipid
added augmented (right panel in Figure S2-B). Fitting of the experimental values to
function [1], disclosed \(K\) values higher than \(10^7\) for AISpreTM in agreement with the free
energy values calculated for a structured sequence (Table 1). By contrast no changes in AIS
Trp emission were detected in the presence of vesicles (Figure S2). Thus, the MPER AIS
stretch alone displayed no significant affinity towards membranes, while extending the
sequence to include the complete PreTM augmented the interfacial affinity so as to produce
almost complete incorporation into vesicles at lipid concentrations above 100 \(\mu\)M (>95 %
of added peptide associated with membranes). Together, the results confirm that the
AISpreTM sequence spontaneously inserts into electrically neutral vesicles, adopting an
equilibrium \(\alpha\)-helix structure therein.

The blocking of the AISpreTM-induced membrane restructuring by MAbs 2F5 and 4E10
In a previous contribution we showed that Chol sustained the lytic and fusogenic activities of PreTM peptide measured in a vesicular system (59). Since the membrane activity of PreTM is preserved within the longer sequence, AISpreTM also altered the integrity of Chol-containing vesicles (Figure 2A). Indeed, there was extensive leakage of the aqueous contents from the vesicle in the presence of 1 µM peptide (> 60%), while comparable levels of membrane lipid-mixing required a dose 4 times higher. In these assays AISpreTM incorporation into vesicles was fluorescently monitored as a function of time using the membrane surface-anchored d-DHPE probe. Close contact with inserting peptide residues enables the energy transfer process that results in d-DPHE emission upon excitation of Trp-s at 280 nm. In both cases, kinetic traces of d-DHPE fluorescence increase demonstrated that the process of peptide incorporation into vesicles occurred within a few seconds (dotted traces). By contrast and in accordance with the rearrangement of peptide monomers at the membrane surface (59), the kinetics of leakage and fusion were slower and both processes required several minutes to stabilize (continuous traces).

The fast incorporation and slower membrane restructuring induced by AISpreTM allowed the capacity of MAb 2F5 and 4E10 to block MPER membrane activity to be compared by assessing the effect of adding the MAbs on AISpreTM-induced leakage or fusion (Figure 2B, top and bottom panels, respectively). Accordingly, the peptide was added to a stirred solution of lipid vesicles (1) and the Mab was injected into the mixture after the peptide has incorporated into the vesicles according to the d-DHPE assay (2). Consistent with the recognition of membrane-bound species under these conditions, the 4E10 Mab efficiently interfered with both leakage and fusion (right panels), whereas injection of similar doses of the 2F5 Mab had almost no effect these processes (left panels).
The inhibition of membrane-restructuring was dose dependent (Figure 2C) and the percentage inhibition could be inferred from the sudden reductions in the kinetic slopes. For leakage (left panel), IC$_{50}$ values in the order of 30 and 200 nM were observed for the 4E10 and 2F5 MAbs, respectively and thus, the 4E10 MAb was approximately 10 times more potent than the 2F5 MAb in inhibiting AISpreTM-induced leakage. The IC$_{50}$ value for the inhibition of fusion was in the same order in the case of the 4E10 MAb while the 2F5 MAb was unable to completely inhibit fusion within the concentration range tested. Thus, the difference in the capacity to inhibit AISpreTM-induced fusion was even higher.

**Stable AISpreTM-mediated MAb-bilayer association**

In order to confirm the in-membrane recognition of the inserted AISpreTM, we evaluated MAb-liposome association under comparable experimental conditions (i.e., stirred, diluted liposome samples). The existence of a stable association of the antibody to the surface of vesicles was first assessed by electron microscopy (Figure 3). Samples of control untreated POPC:Chol vesicles showed heavily stained membrane edges that clearly contrasted with the surrounding medium (Figure 3A), and a similar morphology was displayed by vesicles incubated with AISpreTM alone at concentrations that induced leakage from individual vesicles but not fusion (Figure 3B). However, incubation of these peptide-containing vesicles with the 4E10 (Figures 3D and 3E) or 2F5 (Figures 3G and 3H) MAbs produced vesicles with electrondense particles and punctate edges, which were consistent with antibody molecules protruding from the bilayer surface. These antibody particles could not be detected when POPC:Chol vesicles devoid of the peptide epitope
were exposed to the MAbs (Figures 3C and 3F). Thus, the morphological data supports a physical interaction of the MAbs with the lipid bilayer surrounding the vesicles in the presence of AISpreTM.

This association of the MAbs with the vesicles was next monitored by FACS set up to simultaneously detect liposome and antibody fluorescent signals (Figure 4, see Materials and Methods). As judged from the appearance of a main antibody-stained vesicle population (Figure 4A), both Mabs efficiently associated with POPC:Chol vesicles that contained AISpreTM (>90% of the vesicles were labeled with fluorescent antibody upon incubation with 50 µg/ml MAb). In contrast, when the MAbs were incubated under comparable conditions with POPC:Chol liposomes devoid of peptide, the antibody-stained vesicles constituted a minimal fraction (<5% of the vesicle population).

Hence, this flow cytometry analysis confirmed that AISpreTM mediated the stable association of MAbs with the lipid bilayer. As a consequence, we performed further cytometric measurements to quantitatively compare the capacity of both MAbs to stably associate with vesicles doped with AISpreTM (Figure 4B). These measurements served to ascertain whether the weaker inhibition induced by the 2F5 Mab was due to reduced binding, or whether the binding of this MAb to AISpreTM did not effectively block membrane activity. Titration data confirmed that the 4E10 MAb associated with vesicles at lower concentrations (Figure 4B). Moreover, in both cases a population of MAb-stained vesicles could be recovered at the MAb concentrations that inhibited leakage. Together, these results support the notion that stable association of the 2F5 and 4E10 MAbs with peptide-containing POPC:Chol vesicles correlates with their capacity to inhibit AISpreTM-induced membrane-restructuring.
Specificity of membrane-bound AISpreTM recognition and blocking

Our previous results are consistent with the in-situ recognition and blocking of membrane-inserted AISpreTM and in addition, they suggest that the 4E10 Mab is more potent in mediating both effects. In order to demonstrate the involvement of epitope recognition in these processes, we analyzed sequences in which Ala substituted for Asp9 and Lys10 (AISpreTM(9,10)A), or Trp17 and Phe18 (AISpreTM(17,18)A) to control for the specificity of the interaction of the 2F5 and 4E10 Mabs, respectively (Figure 5). These substitutions were inspired by the alanine-scanning mutagenesis that pinpointed these residues as crucial for the neutralizing activity of the 2F5 and 4E10 Mabs (87). The AISpreTM(9,10)A and AISpreTM(17,18)A sequences were therefore designed to produce the strongest effect on recognition by only one of the two MAbs, while reducing the effect on the membrane restructuring efficiency of the peptide variants as much as possible.

ELISA studies confirmed the failure of the 4E10 MAb to recognize AISpreTM(17,18)A while it efficiently bound to plaques coated with the AISpreTM(9,10)A variant (Figure 5A). Likewise, the 2F5 Mab bound to plates coated with AISpreTM(17,18)A but not to plates coated with AISpreTM(9,10)A. We then evaluated the capacity of these MAbs to block the vesicle permeabilization induced by these variants in PC:Chol vesicles (Figure 5B). Both MAbs were added at doses that almost completely arrested the process induced by the wild-type AISpreTM (insets). In accordance with the specific recognition in membranes, the 4E10 MAb blocked AISpreTM(9,10)A-induced but not AISpreTM(17,18)A-induced leakage. Conversely, the 2F5 MAb induced the opposite effect.
and specifically, it arrested AISpreTM(17,18)A-induced leakage while it exerted almost no effect on the process induced by the AISpreTM(9,10)A variant. The results of a FACS analysis (Figure 5C) confirmed that the membrane blocking activity correlated with the MAb-vesicle association. Thus, vesicles labeled with fluorescent antibodies were recovered from a mixture of AISpreTM(17,18)A/MAb2F5 or AISpreTM(9,10)A/MAb4E10 but not from samples in which AISpreTM(17,18)A/MAb4E10 or AISpreTM(9,10)A/MAb2F5 were combined.
Discussion

Gp41 MPER harbors the linear epitopes recognized by the broadly neutralizing 2F5 and 4E10 Abs (NAbs) (15, 82). Consequently, MPER-based peptidomimetics have been thoroughly scrutinized as immunogenic candidates (43, 86). Soluble, conformationally constrained synthetic derivatives (7, 30, 44, 73) or MPER residues inserted into host protein loop sequences (20, 22, 28, 36, 48, 55) almost invariably induce the recovery of epitope-recognizing responses devoid of virus-neutralizing activity. The finding that the MPER PreTM sequence might stably insert and reside at the membrane interface (58, 64, 70, 72), and that both NAb-s possess long CDR3 loops of heavy chains (32, 34, 88) bearing exposed hydrophobic-at-interface residues (19, 51, 62), suggest that recognition in the membrane might correlate with neutralizing activity. These observations motivated the analysis of the immunogenic properties of lipid-bound spike versions reconstituted on solid-phase liposomes or present in the context of virus-like particles (VLPs) (26, 31, 42, 55). Even though both NAbs bound with higher affinity to envelope glycoproteins in membranes (51), the immune responses recovered after immunization with these specimens were mainly directed against spike regions outside the MPER (31, 42, 55), which underlines the relative low immunogenicity of this gp41 stretch (84).

A yet unexplored alternative strategy to circumvent this problem might consist in the use of formulations combining MPER-derived sequences (synthetic peptides) and model membranes (liposomes). Implementation of such a strategy requires the prior characterization of the factors conditioning the effective interaction of NAbs with MPER sequences embedded in liposomal membranes. For instance, the physicochemical
conditions at the membrane-interface region might modulate the recognition process by affecting MPER penetration and/or its surface aggregation, as well as by restricting stable NAb-membrane association. In addition, the transfer into the membrane interface imparts a defined secondary structure to protein sequences that integrate into the lipid bilayers ("partitioning-coupled folding") (35, 78). The viral external membrane is enriched in Chol and sphingomyelin lipid rafts (2, 3, 12) that modulate membrane interactions of MPER-derived synthetic peptides (39, 59, 75, 77). Thus, these lipids might be anticipated to play a role in the HIV-1 neutralizing activity. Indeed, the 4E10 Mab efficiently recognized a PreTM peptide in phosphatidylcholine membranes containing cholesterol, but not in those containing sphingomyelin (37). Moreover, the 4E10 bound the PreTM in situ, i.e., without detaching from the membrane (37). The 4E10 Mab was formally shown to recognize membrane-bound peptide and induce the extraction of membrane-buried W672 and F673 residues, thereby defining the molecular mechanism for this effect (72). Accordingly, the binding of the 2F5 and 4E10 MAbs to peptide-lipid conjugates was better defined by a two-step (encounter-docking) conformational change, while binding to linear epitopes followed a simple adsorption Langmuir model (1).

It has also been proposed that as a part of their neutralization mechanism, NAb binding to membrane-inserted epitopes would interfere with the fusogenic activity of aromatic-rich MPER sequences by specifically blocking their membrane restructuring activity (37, 72). We have tested this hypothesis using AISpreTM, a peptide that combined the extended 2F5 and 4E10 epitopes (13, 54). AISpreTM adopted an $\alpha$-helical structure in the membrane environment, which facilitates its spontaneous incorporation from aqueous solution into membrane particles in which both epitopes were exposed at the bilayer interface. This
peptide also retained the capacity of PreTM to interact with and induce membrane restructuring of Chol-containing vesicles. The experiments carried out here formally prove that both Abs can block the membrane restructuring activity of AISpreTM, and that this capacity is correlated with specific epitope recognition and their stable association with membranes.

By FACS and electron microscopy we clearly show that the MAbs do not associate with electrically neutral vesicles devoid of AISpreTM. Thus, the correlation between blocking AISpreTM and stable MAb-vesicle association, and the dependence of both effects on specific epitope recognition might be key features of a common mechanism. By contrast, we previously reported the direct, weak association of MAbs to electrically neutral vesicles devoid of peptide (62). This discrepancy may be explained by having previously measured the association of the MAb to immobilized, highly-curved vesicles. The mean diameter of those vesicles is in the range of the bilayer width, which results in the expansion of the external membrane monolayer and that may in turn facilitate the insertion of the MAbs incubated with the immobilized vesicles over long periods of time (41).

The capacity of insertion, in combination with overall positively charged paratope surfaces, is also likely to underlie direct binding to anionic phospholipid bilayers, particularly to those containing cardiolipin (CL) and phosphatidylinositol phosphate (1, 8, 11, 27, 61). Putative autoantigen CL mimicry by MPER and the subsequent control of broadly reactive anti-MPER producing B cells due to tolerance, have been proposed to explain the inability of the HIV-1 gp160 envelope to induce anti-MPER neutralizing antibodies (27), although this notion has recently been challenged (63). Favorable electrostatic association coupled to easier access to the hydrocarbon core might in part
explain the direct association to CL membranes. This non-specific mechanism would be based on the combination within CL of electrostatic polar-head group repulsion with the non-lamellar conical shape (i.e., a cross-sectional area smaller at the phospholipid polar head-group than at the acyl-chain region) (52). In contrast, access to the bilayer core is more restricted in unstressed bilayers based on cylindrical PC-like phospholipids that contain Chol (17). Our results suggest that MAb insertion in such a context could only be achieved upon specific recognition of membrane-bound epitopes. In summary, it appears that the capacity of the 4E10 and 2F5 MAbs to stably insert into membranes can be observed in anionic bilayers subject to lateral pressure that allows easier access to the hydrocarbon core, or in electrically neutral bilayers upon specific recognition of membrane embedded epitope sequences.

Although both Abs were capable of recognizing membrane-associated AISpreTM and blocking its restructuring activity, the 2F5 MAb was clearly less efficient. The ELISA results indicated that this lower capacity could not be explained by the reduced affinity of the 2F5 MAb for AISpreTM immobilized in plates. Thus, we propose two possible explanations for the differences observed in the presence of lipids. Firstly, assuming that both MAbs act through a common epitope-extraction mechanism, membrane-interface partitioning-coupled folding might more severely restrict recognition by the 2F5 Mab (model in supplemental Figure S3). Since the MPER residues K665 and W666 are deeply buried in the 2F5 paratope (51) but they are inserted at the level of the interface when membrane-bound (72), these residues must be extracted or reoriented upon 2F5 MAb binding. The crystal structure of the Fab-bound 657EQELLELDKWASLW670 2F5 epitope reveals an N-terminal extended region comprising EQELLEL residues, which is followed
by two overlapping type I β-turns, one between residues DKWA and the other between residues WASL (51). In contrast, the NMR structure in DPC micelles identifies a helical conformation for the 664DKWASLW670 2F5 epitope stretch (72), which according to our predictions and IR determinations can be extended to include the complete 2F5 epitope. Thus, upon 2F5 MAb binding, the helix organized at the interface has to transit through unordered chain conformations before fitting into the Fab 2F5-bound structure constrained by distinct dihedral angles (Figure 3S, left). The energy required to unfold the 2F5 epitope chain at the interface might reach \( \approx 6 \text{ Kcal mol}^{-1} \) (35). As such, the extraction of K665/W666 residues is associated with the added penalty of the change in secondary structure at the membrane-interface, which adds to the entropic cost of desorption from the mobile hydrophobic environment and further immobilization within the paratope. By contrast, both the membrane-bound and the Fab 4E10-bound epitope sequences are mainly helical (18, 72). In this case, MAb binding results in desorption-reorientation while overall chain conformation is retained (Figure 3S, right).

Alternatively, the membrane-inserted AISpreTM peptide might not accurately represent the native gp41 structures recognized by the 2F5 and/or 4E10 MAbs. Indeed, when assayed under comparable conditions the 2F5 MAb consistently shows higher affinity and neutralizing potency than the 4E10 Mab (1, 9). Moreover, the 2F5 MAb efficiently blocked cell fusion and infection of Moloney murine leukemia virus bearing the 2F5 epitope inserted in the surface subunit, supporting the notion that membrane proximity is not involved in neutralization by 2F5 (53). Thus, the comparatively lower affinity of the 2F5 Mab reported here could be exclusively attributed to the recognition of membrane-inserted MPER states. This phenomenon might in turn reflect the more complex structure of the
native 2F5 epitope than that recreated by the membrane-inserted amphipathic-at-interface
AIS helix (46).

ACKNOWLEDGEMENTS

This study was supported by MEC (BFU2005-06095/BMC to JLN and BFU2007-62382/BMC to JMV) and the University of the Basque Country (UPV 042.310-13552/2001 and DIPE06/11) (JLN). M.L. and R.M. received pre-doctoral fellowships from the Basque Government and the University of the Basque Country, respectively.
Figure legends:

**Figure 1:** Gp41 MPER sequence, derived peptides and predicted membrane effects. A) Membrane proximal and transmembrane sequences of the HIV-1 gp41 integral subunit (sequence and numbering are derived from the prototypic HXBc2 isolate). The gray continuous line on top represents the average hydropathy plot calculated for a window of 5 amino acids using the WW hydrophobicity scale at membrane interfaces (81). The red dotted line is the mean WW moment (window of 11 amino acids) calculated for a fixed $\delta = 100^\circ$ (helical periodicity) and hydrophobicity-at-interface scale (58). Full 2F5 and 4E10 epitopes are shown in red and green, respectively. Residues belonging to the transmembrane domain are shown in blue. The box below displays the peptide sequences used in this study. MPER designates the peptide used by Sun et al. (72). B) Left: Predicted membrane topology for the sequences depicted in the previous panel. The MPER 656-683 region is shown to consist of two segments: an amphipathic sequence (red) followed by a fully interfacial stretch (green). The tilt of the N-terminal amphipathic helix is based on the bipartite NMR structure observed in DPC micelles (72). Insertion of this interfacial element into the external monolayer of the virion membrane might be required to disrupt interactions between lipid molecules and be directly involved in the destabilization of membrane integrity. The differential surface increase of the external membrane monolayer might also contribute to membrane deformation and thinning. Right: Extraction of the epitope sequences by 2F5 or 4E10 would result in bilayer architecture stabilization, thereby interfering with fusion.
Figure 2: AISpreTM-induced membrane-restructuring in POPC:Chol (2:1 mol:mol) vesicles and its inhibition by the 2F5 and 4E10 MAbs. A) In left and center panels, continuous lines correspond to the kinetic traces of leakage (left) or fusion (center) upon AISpreTM injection into stirred solutions of LUV (indicated by the arrows) at a final peptide concentration of 1 or 4 µM, respectively. The dotted traces follow the incorporation of AISpreTM into the vesicles monitored through energy transfer from peptide tryptophans to membrane-residing d-DHPE. The right panel displays the percentage of AISpreTM-induced leakage (circles and continuous lines) and lipid-mixing (squares and dashed lines) after a 30 minute incubation with vesicles plotted as a function of the peptide concentration. L-B arrow indicates the condition selected to measure MAb-induced blocking of leakage and MAb binding to vesicles, and the F arrow is that selected to measure MAb-induced blocking of fusion. The final lipid concentration was 100 µM in all cases. B) Inhibition of ANTS leakage (top) and mixing of vesicle lipids (bottom). LUV suspensions (100 µM lipid) were treated with 1 or 4 µM AISPreTM, respectively and subsequently they were supplemented with 10 µg/ml of the 2F5 or 4E10 MAbs (addition times indicated by "1" and "2", respectively). Dotted traces correspond to the controls in the absence of antibody. C) Dose dependency of AISpreTM-induced ANTS leakage and fusion inhibition by the 4E10 MAb (circles and continuous line) or 2F5 MAb (squares and dotted line). The initial rates were determined as the increase in fluorescence during the 20 seconds that followed MAb addition. Inhibition percentages were plotted as a function of the MAb concentration and the data are represented as the mean ± S.D. of 3 (leakage) and 4 (fusion) independent experiments.
**Figure 3:** Physical MAb-bilayer association as seen by electron microscopy in POPC:Chol (2:1) LUV. Panels A and B correspond to electron micrographs of control vesicles devoid of peptide and AISpreTM-containing vesicles (peptide-to-lipid mole ratio, 1:100), respectively. D-E and G-H show AISpreTM containing vesicles incubated with the 4E10 or 2F5 MAbs, respectively. These samples consist of vesicles (1 mM) incubated for 5 minutes with AISpreTM and then supplemented with 50 µg/ml MAb before staining with uranyl acetate. The corresponding C and F panels display control samples devoid of the AISpreTM peptide. Bars = 100 nm.

**Figure 4:** Stable MAb-vesicle association as determined by flow cytometry. A) The 4E10 and 2F5 MAbs (50 µg/ml) were incubated with a stirred solution of f-DHPE-labeled LUV (250 µM) under experimental conditions that were otherwise similar to those of the leakage blocking (see Figure 2). After 10 minutes, the fluorescently labeled secondary Ab was added and the resulting mixtures were incubated for 5 minutes before being analyzed by flow cytometry. The incubation of AISpreTM-containing POPC:Chol vesicles with the 4E10 and 2F5 Mabs rendered double labeled vesicles (center and top right-hand panels, respectively). This pattern could not be observed in the absence of the MAbs (left panels) or when the MAbs were incubated with bare POPC:Chol vesicles (center and bottom right-hand panels). B) Differential association of the 4E10 and 2F5 MAbs with AISpreTM-containing POPC:Chol vesicles (1:100 peptide-to-lipid molar ratio) as determined by flow cytometry. The 4E10 (blue traces) or 2F5 (red traces) MAb was incubated at the final concentrations (µM) indicated in the panels with AISpreTM-containing LUV under conditions that were otherwise similar to those in the previous panel. Black traces correspond to control samples not incubated with MAb.
**Figure 5:** Dependence of MAb blockage of induced membrane restructuring and MAb-vesicle association on specific epitope recognition. A) The binding of the 2F5 (red squares) and 4E10 (blue circles) MAb to 1 μM AISpreTM(9,10)A (left) or AISpreTM(17,18)A (right) immobilized on ELISA plaques. B) Blocking of leakage induced by the addition of 2 μM AISpreTM(9,10)A (left panel) or 1 μM AISpreTM(17,18)A (right panel) to POPC:Chol vesicles by the 2F5 (red traces) and 4E10 (blue traces) Mab. Black trace corresponds to the control without MAb. The MAbs were added at a concentration of 50 μg/ml. The insets display the blocking induced by the same concentration of the 2F5 (left) or 4E10 (right) MAb on the leakage induced by parental AISpreTM. C) Flow cytometry determination of the association of the 2F5 (red) and 4E10 (blue) MAb with POPC:Chol vesicles bearing AISpreTM(9,10)A (left panel) or AISpreTM(17,18)A (right panel). MAbs were incubated with the vesicles at a concentration of 30 μg/ml.
TABLE 1. Energetics of the interaction with the membrane interface and penetration into lipid monolayers of MPER-derived sequences.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Gp160 Number.a</th>
<th>$\Delta G_{(wiu)}$ b</th>
<th>$\Delta G_{(wif)}$ b</th>
<th>$\Delta G_{(obs)}$ b</th>
<th>$\pi_{ex}$ c</th>
</tr>
</thead>
<tbody>
<tr>
<td>AISpreTM</td>
<td>656-683</td>
<td>-2.8</td>
<td>-14</td>
<td>$&lt;-9.5$</td>
<td>43</td>
</tr>
<tr>
<td>PreTM</td>
<td>664-683</td>
<td>-8.1</td>
<td>-16.1</td>
<td>$&lt;-9.5$ d</td>
<td>37 d</td>
</tr>
<tr>
<td>AIS</td>
<td>656-671</td>
<td>+4.1</td>
<td>-2.3</td>
<td>$&gt;0$</td>
<td>28</td>
</tr>
<tr>
<td>MPER$^e$</td>
<td>662-683</td>
<td>-6.7</td>
<td>-15.5</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

aNumbering based on the HXBc2 reference isolate.

bThe free energies (Kcal/mol) for partitioning from water into membrane interfaces calculated for unfolded (wiu) and folded (wif) peptide chains are compared with experimentally determined values (obs).

cCritical pressures (mN/m) for insertion into lipid monolayers ($\pi_{ex}$) have been derived from the data displayed in Figure S1-A.

dValues as reported in (58).

eMPER-derived peptide sequence used by Sun et al. (72).
References:


Immunogenicity of recombinant human immunodeficiency virus type 1-like particles expressing gp41 derivatives in a pre-fusion state. Vaccine 25:5102-14.


39. Lorizate, M., N. Huarte, A. Saez-Cirion, and J. L. Nieva. 2008. Interfacial pre-
transmembrane domains in viral proteins promoting membrane fusion and fission. Biochim Biophys Acta.


64. Schibli, D. J., R. C. Montelaro, and H. J. Vogel. 2001. The membrane-proximal


Immunity 28:52-63.


A

\[ \Delta G \text{ (Kcal mol}^{-1}\text{)} \]

W-W Hydrophobicity

W-W Moment

655KNEQELLELDKWasLWNWFNITNWLYIKLFIMIVGGLVGLRIVFAVLSVYNRVRQGYSPLSFQ719

\[ \text{AISpreTM: } 656\text{NEQELLELDKWasLWNWFNITNWLYIK683} \]

\[ \text{AISpreTM(9,10)A: } 656\text{NEQELLELAAWasLWNWFNITNWLYIK683} \]

\[ \text{AISpreTM(17,18)A: } 656\text{NEQELLELDKWasLWNAANITNWLYIK683} \]

PreTM:

664DKWasLWNWFNITNWLYIK683

AIS:

656\text{NEQELLELDKWasLWN671} \]

MPER:

662\text{ELDKWasLWNWFNITNWLYIK683} \]

B

2F5

4E10

2F5

4E10