Recognition and Blocking of HIV-1 gp41 Pre-transmembrane Sequence by Monoclonal 4E10 Antibody in a Raft-like Membrane Environment*

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The conserved 664DKWASLWNFNITNWLWYIK683 (preTM) sequence preceding the transmembrane anchor of human immunodeficiency virus (HIV-1) gp41 glycoprotein subunit is accessible to the broadly neutralizing 4E10 antibody and, therefore, constitutes a potential target for vaccine design. Recently reported structural data are compatible with preTM insertion into the viral external membrane monolayer in the gp41 pre-fusion state (Zhu, P., Liu, J., Bess, J., Chertova, E., Lifson, J. D., Grise´, H., Ofek, G. A., Taylor, K. A., and Roux, K. H. (2006) Nature 441, 847–852). Here we demonstrate that the broadly neutralizing 4E10 antibody is able to specifically block the membrane-restructuring activity of a peptide mimic inserted into membranes. Recognition and restructuring blocking occurred in the presence of cholesterol, whereas transmembrane versions as those promoted in 1-palmitoyl-2-oleoylphosphatidylcholine:sphingomyelin mixtures could not be effectively arrested. Spectrofluorimetric assays using rhodamine-labeled peptides revealed that recognition correlated better with pore-formation blocking than with membrane-fusion inhibition. The capacity of the antibody to recognize preTM peptides in a raft-like environment was further corroborated employing planar-supported lipid layers and fluorescence microscopy. These data support that membrane-bound epitope recognition by 4E10 results in clustering reorganization of preTM at the membrane interface. We propose that this process might interfere with the formation of fusion-competent complexes at the low spike densities existing in the HIV-1 membrane. This work comprises the first experimental report on a lipid-modulated antibody capacity to bind a membrane-bound epitope sequence and arrest its restructuring activity.

Only three broadly neutralizing anti-HIV-14 monoclonal antibodies (mAbs), 2F5, 4E10, and Z13, have been identified to react with the membrane-integral gp41 Env subunit that promotes viral-cell fusion (for a recent review, see Ref. 1). These three antibodies recognize epitopes within the conserved aromatic-rich domain that precede the gp41 transmembrane anchor (2–7). mAb4E10 bears the broadest activity, showing the capacity of neutralizing all tested M group clades and newly transmitted virus isolated from infected patients (8–11). Thus, there exists a considerable interest in unraveling the mechanistic basis of the broad neutralizing activity of mAb4E10.

The epitope core 671NWF(D/N)IT676 recognized by mAb4E10 locates at the center of the gp41 664DKWASLWNFNITNWLWYIK683 sequence. This sequence has been defined as a distinct domain according to its interfacial hydrophobicity (designated as pre-transmembrane (preTM)) (2–4) and also attending to its implication in fusion and neutralization processes (designated as a membrane-proximal external region) (1, 5–7). Pioneering mutational analyses by Salzwedel et al. (5, 6) and biophysical determinations by our group (2–4) suggested that preTM is instrumental for successful gp41-mediated fusion. Specifically, this domain would be involved in the expansion of the fusion pores (6, 12). However, the structural grounds sustaining this activity are poorly understood due in part to the fact that a gp41 ectodomain atomic structure including the membrane-proximal region is not available at present. Our analyses predicted a membrane interfacial location of the sequence as a monomer (2, 4). NMR studies by Schibli et al. (13) further demonstrated that the preTM peptide adopts a well defined helical conformation in the membrane-like environment provided by dodecylphosphocholine micelles. In addition, nuclear Overhauser effects between side chains and polar head groups suggested that aromatic residues would position within the interfacial bilayer region (Fig. 1B).

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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 51, pp. 39598 –39606, December 22, 2006 © 2006 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.

*This study was supported in part by the Spanish MCyT Grants BFU2005-06095/BMC (to J. L. N.) and BIO2006-03130 (to J. P.-G.) and by University of the Basque Country Grant 042.310-13552. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1Recipient of a pre-doctoral fellowship of the Basque Government.

2Supported by Community of Madrid Grant CAM-S-505-MAT-283 and FP6 of European Union Grant MEST-CT-2004-007931.

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4The abbreviations used are: HIV-1, human immunodeficiency virus type-1; Chol, cholesterol; mAb, monoclonal antibody; MBCD, methyl-β-cycloex- trin; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; preTM, pre-transmembrane; SPM, sphingomyelin; LUV, large unilamellar vesicles; NBD, 7-nitro-benz-2-oxa-1,3-diazol-4-yl; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid sodium salt; DPX, p-xylenebis(pyridinium).
pholipid bilayers depending on peptide load and lipid composition (3, 4). Subsequent x-ray diffraction data obtained by Cardoso et al. (14) have indeed revealed a helical structure for a 13-residue preTM derivative in complex with Fab4E10. Finally, preTM appears to bind directly cholesterol (Chol) through its C-terminal cholesterol recognition/interaction amino acid consensus WYIK sequence (15), a phenomenon that might condition gp41 fusogenic function (16).

The physiological relevance of the predicted preTM interfacial location has recently received support from the cryoelectron microscopy tomography study by Roux and co-workers (17). This study has revealed that gp41 stalk regions project as “legs” from the trimeric Env complex in intact virions, with their “feet” just above the plane of the envelope (tripod-like structure). Docking of the Fab-bound atomic structures into the surface model suggests that Env native preTM would actually locate at the viral surface, inserted as a monomer in parallel to the external membrane monolayer. Thus, it is inferred that mAb4E10 bears the capacity to recognize native-like epitope sequences associated to the viral membrane interface.

In this work we test this prediction by assessing the capacity of mAb4E10 to recognize and block preTM peptides inserted into lipid vesicles and monolayers. Specifically, we have compared the effects on recognition of sphingomyelin and cholesterol, the two raft-lipids postulated to be predominant at the virion external monolayer (18–20). Our studies support the notion that 4E10 paratope is structurally designed to allow recognition of preTM species stabilized by cholesterol at the virion external membrane-interface. Moreover, we propose that recognition might render cross-linked spikes incompetent for fusion. These observations might be important for the design of immunogens aimed at recovering 4E10-like protective responses.

**EXPERIMENTAL PROCEDURES**

*Materials—* The sequences DKWASLNWFNITNWLYIK (preTM), representing the pre-transmembrane stretch of HIV-1 (BH10 isolate) gp41, and its fluorescent derivative (Rho-preTM), labeled with rhodamine at its N terminus, were synthesized by solid-phase synthesis using Fmoc chemistry as its C-terminal carboxamido and purified by high performance liquid chromatography at the Proteomics Unit of the University Pompeu-Fabra (Barcelona, Spain). Peptide stock solutions were prepared in dimethyl sulfoxide (Me₂SO) (spectroscopy grade), and concentrations were determined by bicinchoninic acid microassay (Pierce). Neutralizing antibody expressing hybridomas were generated by a combined polyethylene glycol electrofusion of peripheral blood mononuclear cells of HIV-infected non-symptomatic patients (21). Neutralizing antibodies 2F5 and 4E10 were produced in recombinant Chinese hamster ovary cells after a subclass switch to IgG1 (9, 22). 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC), Chol, sphingomyelin (SPM), and the fluorescent probes N-(7-nitro-benz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (N-(NBD)-phosphatidylethanolamine) and N-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine were purchased from Avanti Polar Lipids (Birmingham, AL). The N-(5-dimethylaminonaphtalene-1-sulfonil)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, 8-aminonaphtalene-1,3,6-trisulfonic acid sodium salt (ANTS), and p-xylenebis(pyridinium)bromide (DPX) fluorescent probes and the goat anti-human IgG antibody labeled with Alexa-Flour 488 were from Molecular Probes (Junction City, OR). Methyl-β-cyclodextrin (MBCD) and sphingomyelinase (from Bacillus cereus) were from Sigma.

Production of Lipid Vesicles—Large unilamellar vesicles (LUVs) were prepared following the extrusion method of Hope et al. (23) in 5 mM Hepes, 100 mM NaCl (pH 7.4) buffer. Lipid concentrations of liposome suspensions were determined by phosphate analysis (24). Extraction of cholesterol from the vesicles was performed using MBCD as previously described (25, 26).

Fluorimetric Assays—Release of vesicular contents to the medium was monitored by the ANTS/DPX assay (27). 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 in a Sephadex G-75 column eluted with 5 mM Hepes, 100 mM NaCl (pH 7.4). Osmolarities were adjusted to 200 mosmol 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 0; 100% leakage was the fluorescence value obtained after the addition of Triton X-100 (0.5%, v/v).

Membrane lipid mixing was monitored using the resonance energy transfer assay, described by Struck et al. (28). The assay is based on the dilution of N-(NBD)-phosphatidylethanolamine and N-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine. Dilution due to membrane mixing resulted in an increased N-NBD-phosphatidylethanolamine fluorescence. Vesicles containing 0.6 mol % of each probe were mixed with unlabeled vesicles at a 1:4 ratio (final lipid concentration, 0.1 mM). The NBD emission was monitored at 530 nm with excitation wavelength set at 465 nm. A cutoff filter at 515 nm was used between the sample and the emission monochromator to avoid scattering interferences. The fluorescence scale was calibrated such that the zero level corresponded to the initial residual fluorescence of the labeled vesicles, and the 100% value corresponded to 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.

Kinetics of peptide partitioning into the membrane interface was evaluated measuring energy transfer from Trp residues to the surface fluorescent probe N-(5-dimethylaminonaphtalene-1-sulfonil)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine in as in Ruiz-Argüello et al. (29). In brief, 6 mol % of N-(5-dimethylaminonaphtalene-1-sulfonil)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine probe was included in the target vesicle composition, and its emission was collected in time at the wavelength of 510 nm. The excitation wavelength was that of the Trp residue (280 nm).

Monolayer Penetration—Surface pressure was determined in a fixed-area circular trough (μTrough S system, Kibron, Helsinki). Measurements were carried out at room temperature and under constant stirring. The aqueous phase consisted of 1
gp41 preTM Sequence Blocking by mAb4E10

A

preTM: 664DXWASLWNWFNTWNLWYI683

4E10

B

FIGURE 1. A, average hydrophathy-at-interface for the HIV-1 gp41 ectodomain including the transmembrane region (residues plotted, 510–711 of gp160 precursor in BH10 isolate, GenBank™ M15654) and the heavy chain of mAb4E10 (top and bottom plots, respectively). A window of 11 amino acids (aa) was used with the hydrophobicity scale at membrane interfaces of Wimley and White (41). The sequence designated above is derived from the positive gp41 peak in black and corresponds to the preTM-representative peptide. Residues spanning the 4E10 core epitope are indicated by the bar. Residues in blue span the membrane-interface embedded structure depicted in panel B. Black peaks in the 4E10 plot designate stretches with a favorable tendency to partition into membrane interfaces within CDRs H2 and H3. B, front and side-view structures of the preTM stretch encompassing W670–W678 sequence, immersed into membrane interfaces (blue chain) or bound to Fab4E10 (green chain). To guide their relative orientation, both structures include side chains of aromatic Trp-670, Trp-672, Phe-673, and Trp-678. In addition, the side chain of Phe-673 is colored in red. Solid ribbons correspond to the Fab CDR L3, H2, and H3 loops as indicated and are displayed to place the epitope binding cavity. Also for the matter of comparison, Trp-678 and W670 residues in both structures have been positioned roughly at the same level within the membrane interface (MI). HC and ext designate hydrocarbon core and external bilayer regions, respectively. Structures derived from 1JAU (13) and 1TZG (14) PDB entries were rendered with the Swiss-PDB viewer program.

RESULTS

Fig. 14 displays the average interfacial hydrophobicities of HIV-1 gp41 ectodomain and mAb4E10 heavy chain (top and bottom plots, respectively). The gp41 sequence displays a prominent hydrophobic region comprising the aromatic-rich pre-TM 664–683 sequence. The core 4E10 epitope sequence lies in the center of the preTM stretch. Therefore, mAb4E10 is predicted to recognize a sequence that may stably reside at membrane interfaces. The mAb4E10 plot below displays two hydrophobic peaks (colored in black) that correspond to loop sequences containing exposed aliphatic and aromatic residues in the heavy chains (14). These two loops have been postulated to establish direct interactions with the membrane, a process that might help with recognition of the membrane-inserted epitope stretch (14, 32).

Fig. 1, panel B, compares the atomic structures of a preTM-derived sequence resolved by Cardoso et al. (14) in complex with Fab4E10 (green chain) or immersed into dodecylphosphocholine micelles according to the NMR-based model proposed by Schibli et al. (13) (blue chain). The helical structure is the main conformation in both cases. However, the relative orientation of the aromatic residues is different. Specifically the Phe-673 (red side chain) is buried into a hydrophobic pocket of the Fab binding site, whereas the side chain of this residue projects toward the hydrocarbon core of the bilayer in the membrane-interface residing species. The different orientations and insertion levels suggest that the potential recognition of the membrane interface residing preTM by mAb4E10 should result in blocking of its membrane restructuring capacity.

mAb4E10 Effects on PreTM-induced Membrane Restructuring—Results displayed in Figs. 2 and 3 confirmed that mAb4E10 was able to block PreTM-induced membrane restructuring in Chol-containing vesicles. We have previously reported that the PreTM peptide perturbs vesicles made of lipids that resemble the raft-like composition of HIV-1 external membrane monolayer (3, 18–20). In particular, PreTM peptide partitions almost quantitatively into POPC:SPM:Chol (1:1:1) vesicles and induces the release of the encapsulated solutes (3). As shown in Fig. 2A, the addition of mAb4E10 alone to these vesicles did not induce any appreciable ANTS release, suggesting that the antibody was not able to destabilize the lipid bilayer on its own. However, antibody addition before PreTM effi-
Inhibition of pore formation with mAb4E10.

A, inhibition of PreTM-induced POPC:SPM:Chol (1:1:1) LUV permeabilization (ANTS leakage) by preincubation with mAb4E10 (a trace). mAb4E10 (50 μg/ml) was added at the time indicated by the arrow. The control in the absence of antibody (b trace) was measured at 1:50 peptide-to-lipid molar ratio and 100 μM lipid. B, dose dependence of the previous process measured at 1:100 (squares and dotted line) or 1:500 (circles and continuous line) peptide-to-lipid ratios. C, inhibition of ongoing ANTS leakage by mAb4E10 addition. At the time indicated by the arrow 50 μg/ml of mAb4E10 (a trace) were added to PreTM-treated POPC:SPM:Chol (1:1:1) LUV. Superimposed b and c traces correspond to the addition of 50 μg/ml of mAb2F5 and the control without antibody, respectively. Conditions were as in panel A.

The inhibitory effect was dose-dependent and more effective at low peptide-to-lipid ratios, suggesting that the process was directly dependent on antibody recognition (Fig. 2B).

The PreTM-induced ANTS leakage process in POPC:SPM:Chol (1:1:1) vesicles satisfies the prerequisites for permeabilization occurring via lytic pore formation (3). Accordingly, ANTS leakage kinetics reproduced the time-course required for self-aggregation and pore assembly at the membrane surface. Thus, this process may comprise a potential means for testing antibody recognition of membrane-bound species. mAb4E10 addition to vesicles undergoing permeabilization also arrested the pore formation (Fig. 2C). The PreTM peptide lacks the complete ELDKWA epitope sequence required for efficient mAb2F5 recognition, and this antibody could not arrest the leakage process (b trace). This observation reinforces the idea that blocking of membrane-bound species competent for membrane destabilization depended on specific 4E10 epitope recognition.

Membrane restructuring could not be completely arrested in the previous samples, suggesting the existence of a peptide fraction not accessible to the antibody. In our previous contribution we showed that raft lipids SPM and Chol promoted different PreTM species in membranes (3). When mixed with POPC, SPM sustained pore-formation, whereas Chol also promoted fusion activity, a phenomenon that we interpreted as indicative of transmembrane and interfacial topologies. Therefore, the role of each raft lipid in the blocking-at-membrane processes was next analyzed separately using POPC:Chol (2:1) and POPC:SPM (1:1) vesicles (Fig. 3A).

The time-course of dansylphosphatidylethanolamine fluorescence increase confirmed the immediate incorporation of peptide upon the addition to both types of vesicles, whereas the ANTS leakage kinetics scored the slower assembly of permeating pores at the membrane surfaces. mAb4E10 addition to POPC:Chol vesicles could arrest ongoing permeabilization promoted by membrane-bound species (Fig. 3A, left-hand panel). In sharp contrast, the antibody was not able to inhibit the leakage processes occurring in POPC:SPM vesicles (Fig. 3A, right-hand panel). Again, we note that at the times selected for antibody addition (indicated by the arrows) dansylphosphatidylethanolamine changes indicated that all peptides engaged in membrane-pore formation were actually bound to the vesicles.

Experiments displayed in Fig. 3B corroborated that mAb4E10 blocking effect depended on the presence of Chol. Treatment of POPC:SPM:Chol (1:1:1 mole ratio) LUVs with MBCD almost abolished the capacity of mAb4E10 to block PreTM-induced pore formation (compare the left and middle panels). Thus, the antibody was not able to interfere with the membrane activity of preTM upon specific extraction of the sterol from the membrane. Moreover, results displayed in the right panel supported that SPM did not exert a specific inhibitory effect on the antibody (for instance by directly binding to the 4E10 paratope). Converting SPM into ceramide through sphingomyelinase action is expected to stabilize ceramide-based ordered domains, whereas both Chol and SPM are depleted (Refs. 33 and 34 and references therein). Treatment of POPC:SPM:Chol vesicles with sphingomyelinase actually decreased the capacity of mAb4E10 to block PreTM-induced pore-formation (compare the left and right panels). Thus, we conclude that phase coexistence rather than SPM itself was the factor inhibiting mAb4E10 blocking capacity in the absence of Chol.
Finally, mAb4E10 was also capable of blocking the fusion of POPC:Chol (2:1) vesicles promoted by membrane-bound PreTM (Fig. 3C). The capacities to interfere with ongoing leakage and fusion after virtually all peptide had incorporated into POPC:Chol vesicles suggests the existence of a mAb4E10-epitope recognition process at membrane surfaces that blocks bilayer restructuring.

The dose dependence of this effect is displayed in Fig. 4. mAb4E10 blocked completely PreTM-induced leakage and fusion but was more efficient interfering with the former process. In addition, permeabilization blocking was a dominant effect that effectively developed at binding-site-to-preTM mole ratios of ~1:10. In conjunction, these results support the capacity of mAb4E10 to recognize and block PreTM species embedded into a Chol-containing membrane environment. They further suggest that transmembrane pores formed in POPC:SPM vesicles were poorly recognized by the antibody.

mAb4E10 Recognition and Blocking at Low Doses—Sáez-Cirión et al. (3) found that, in comparison to POPC:Chol binary mixtures, leakage and fusion induced by preTM occurred at much lower peptide doses in POPC:SPM:Chol ternary mixtures that sustain the formation of SPM-Chol-enriched ordered domains. Results in Fig. 5 demonstrate the existence of PreTM recognition and blocking by mAb4E10 under experimental conditions that reproduce more closely the conditions existing at the virion surface, namely, presence of liquid-ordered domains and low spike-to-lipid ratios (17, 20, 35, 36).

In situ recognition of peptides incorporated into POPC:SPM:Chol (1:1:1 mole ratio) LUV at a 1:500 peptide-to-lipid ratio was assayed by scoring the changes in rhodamine intensity upon antibody binding. It was anticipated that binding might cause a reduction in rhodamine intensity due to antibody-induced probe immobilization and/or direct quenching. As expected, mAb4E10 reduced rhodamine emission when added to POPC:SPM:Chol vesicles pre-loaded with Rho-PreTM peptide (a trace in Fig. 5A). Again, mAb4E10 affected Rho-PreTM emission to a lesser extent in POPC:SPM vesicles (b trace in Fig. 5A), a phenomenon consistent with an impaired recognition in this system. The fluorescence intensity reduction observed in

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**FIGURE 3.** Distinct effects of SPM and Chol on the membrane-restructuring blocking capacity of mAb4E10. A, inhibition of ongoing ANTS leakage with mAb4E10. POPC:Chol (2:1) (left-hand panel) or POPC:SPM (1:1) (right-hand panel) LUV suspensions (100 μM lipid) were treated with PreTM (1 μM) and, at the time indicated by the arrow, supplemented with 50 μg/ml of mAb4E10 (a trace). b traces denote the controls in absence of antibody. c traces correspond to dansyl fluorescence increase (arbitrary units) upon PreTM addition. B, specific effects of Chol and SPM on the process. Left-hand panel, control for the inhibition of ongoing ANTS leakage with mAb4E10 (25 μg/ml) as detected in POPC:SPM:Chol (1:1:1) LUV. Middle panel, vesicles were treated with MBCD (15 mM) for 15 min before preTM addition. Right-hand panel, vesicles were treated for 15 min with sphingomyelinase (SPMase; 0.16 units/ml) before preTM addition. Conditions were as in panel A. CTL, control. C, inhibition of intervesicular mixing of lipids with mAb4E10. POPC:Chol (2:1) vesicles undergoing PreTM-induced fusion were treated with 50 μg/ml mAb4E10 (arrow in the a trace). The b trace corresponds to the kinetics of the process in absence of antibody. Conditions were as in panel A.
POPC:SPM:Chol vesicles was dose-dependent (Fig. 5B). Data displayed in Fig. 5C correlate recognition of Rho-preTM at the membrane surface with the inhibition of pore formation (ANTS leakage) and fusion (lipid mixing). Dose dependence of recognition seemed to correlate better with pore-formation inhibition, again suggesting that this cooperative process was more readily affected by antibody binding.

**mAb4E10 Recognition of Membrane Interface-residing preTM**—Recognition of PreTM by mAb4E10 in a raft-like environment was subsequently analyzed employing planar-supported phospholipid layers (3). mAb4E10, but not mAb2F5, used as a control, recognized PreTM inserted into POPC:SPM:Chol (2:1:1) lipid monolayers and induced a decrease of the lateral pressure (Fig. 6A). The reduction was lower when POPC:SPM (1:1) monolayers were used instead. At the concentrations used, mAb4E10 alone did not affect the surface pressure of these monolayers (not shown), suggesting that the observed reduction was a consequence of epitope recognition. Together, these observations were in accordance with mAb4E10-promoted specific desorption of the sequence, as suggested by the models depicted in Fig. 1B.

mAb4E10 interaction with membrane surfaces was revealed through immunofluorescence microscopy of the lipid-film transferred into a solid support (Fig. 6). mAb4E10 associated to PreTM-containing POPC:SPM:Chol monolayers and was detected in dendritic-shaped structures (left-hand panel). These structures were not observed in control samples consisting of antibody-treated monolayers devoid of peptide (middle panel). In comparison, the antibody signal distributed into small fluorescent dots in the POPC:SPM:peptide monolayers (right-hand panel).

To get a better insight into this phenomenon, transferred monolayers that incorporated fluorescently labeled Rho-PreTM were also analyzed. This peptide species concentrates at ordered domain-like structures in POPC:SPM:Chol monolayers (3). Confirming those results, Rho-PreTM was evidenced in this system as fluorescent clusters, often forming short fibrillar structures (Fig. 7A, left-hand panel). In contrast, rhodamine fluorescence distributed into small dots when the peptide was co-dispersed with POPC:SPM (Fig. 7A, right-hand panel). mAb4E10 addition changed Rho-PreTM fluorescence pattern in POPC:SPM:Chol but not in POPC:SPM monolayers (micrographs in panel B). In POPC:SPM:Chol samples, mAb4E10-treated PreTM peptides coalesced into larger clusters display-
ing a dendritic aspect. The actual participation of mAb4E10 in these structures was revealed through immunofluorescence detection and signal co-localization (panel C). Importantly, Rho-preTM films prepared in the absence of lipid showed homogeneous Rho fluorescence but a complete absence of bright spots (not shown). This fact indicates that peptide clusters were not initially present in peptide preparations but only formed upon association with lipids and antibody. In summary, results displayed in Figs. 6 and 7 confirm that mAb4E10 was able to recognize PreTM inserted into monolayers that mimicked the viral membrane external leaflet and also suggest that the antibody sequestered the peptide into large clusters.

**DISCUSSION**

Viral glycoproteins are thought to catalyze fusion between viral envelopes and target cell membranes through the assembly of fusion-competent high order complexes within confined areas of the interacting bilayers (37, 38). HIV-1 acquires its membrane by budding from laterally segregated specific cell membrane platforms or “rafts” enriched in Chol and SPM (18–20). Previous work by our group had shown that aromatic-rich gp41 preTM might exist as a membrane interface-residing sequence whose aggregation state and topology were modulated by those lipids (3). SPM and Chol were proposed to act as fusion co-factors by regulating gp41 activity through the lipid-sensing preTM domain. The experimental results in this chapter add to this notion that (i) the gp41 preTM epitope is recognized by broadly neutralizing mAb4E10 in an envelope-like environment, and (ii) mAb4E10 blocks the Chol-dependent membrane-restructuring processes promoted by preTM.

**4E10 Epitope Presentation in a Membrane Environment**—In line with other gp41-recognizing antibodies, mAb4E10 binds efficiently soluble monomeric peptides that represent the core epitope (14, 39). On the other hand, mAb4E10 is unique because it possesses highly hydrophobic-at-interface variable loops in the heavy chain that contain exposed aromatic residues (i.e. not engaged in direct contacts with the bound epitope) (14, 32). mAb4E10 indeed recognizes with similar apparent affinities epitope peptides directly deposited in enzyme-linked immunosorbent assay plaques or pre-bound to phospholipid vesicles (32). This has been postulated to constitute an adaptation for epitope recognition in a membrane environment. The recent low resolution structure of a pre-fusion SIV spike obtained by Roux and co-workers (17) suggests that preTM is inserted into the interface of the external membrane monolayer, further supporting that native 4E10 epitope recognition occurs in a membrane environment.
Comparison of the preTM atomic structures also suggests that, accompanying epitope recognition, a conformational transition must occur from the species embedded into the membrane to the structure recognized by the antibody in solution (Fig. 1B). Such a transition is predicted to block preTM’s membrane activity. Our results actually confirm that mAb4E10 was able to recognize and thereby arrest the membrane-restructuring effects of the preTM peptide embedded into POPC:Chol vesicles (Fig. 3). Although POPC:SPM-bound preTM was also efficient in pore-formation, mAb4E10 could not effectively block it (Figs. 3 and 5–7). MBCD-induced extraction of Chol from the ternary POC:SPM:Chol mixture confirmed again the dependence of the blocking effect on the sterol (Fig. 3). This is in accordance with the proposed interfacial and transmembrane topologies that the preTM peptide adopts in POPC:Chol and POPC:SPM membranes, respectively, and also supports the specific recognition-blocking of the Chol-dependent interfacial state by mAb4E10. We conclude that mAb4E10 is adapted to specifically recognize the native preTM epitope species occurring at the external monolayer of the HIV-1 virion envelope but not other membrane-associated preTM forms such as those in a transbilayer arrangement (see the model in Fig. 8A).

**PreTM Blocking and mAb4E10 Neutralization Mechanism—** mAb4E10 binding to Chol-dependent preTM forms resulted in leakage and fusion blocking (Figs. 2–5). Titration experiments revealed that mAb4E10 more readily inhibited the leakage process (Figs. 4 and 5). Leakage inhibition also correlated better than fusion inhibition with the recognition by the antibody (Fig. 5). This higher susceptibility to antibody binding probably reflects the cooperative character of pore formation. Earlier pore-model calculations suggested that at least 7–10 preTM monomers participate in the assembly of a permeating pore in Chol-containing bilayers (3). Results in Fig. 4 show that leakage inhibition indeed started when the mole ratio between the antibody binding site and peptide roughly approached 1:10. By comparison, this ratio had to be increased 4–5 times for fusion inhibition triggering.

Microscopic analyses of the supported monolayers indicated that preTM recognition occurs within peptide clusters merged by mAb4E10 (Figs. 6 and 7). This approach also disclosed the overall preTM organization in the different membrane environments. The Chol-dependent state consisted of Rho-preTM clusters (see also Sáez-Cirión et al. (3)). These clusters became more extended and dendritic-shaped upon treatment with the antibody, which was shown to specifically co-localize with the peptide. Conversely, the spotty fluorescence pattern of the SPM-dependent state was consistent in the two transmembrane environments: the Chol-preTM peptide, which was not altered by the antibody. Thus, the abilities of the antibody to interfere with the cooperative processes of leakage and to reorganize the epitope sequence at raft-like membrane surfaces suggest that self-aggregation of native gp41 rather than actual blocking of membrane merging might sustain the mAb4E10 neutralization mechanism.

According to the model depicted in Fig. 8B, mAb4E10 might inhibit gp41-mediated fusion by blocking the oligomerization processes required for fusion-pore expansion (12, 37, 38). In this regard the mechanism of viral neutralization by mAb4E10 could be reminiscent of the broad-spectrum antiviral mechanism recently described by Leikina et al. (40) for the lectin components of the innate immunity system. These authors have shown that the antiviral activity of defensins is based on the capacity of these compounds to block fusion by forming a network of immobilized glycoproteins. Similarly, our data in Figs. 6 and 7 suggest that mAb4E10 could cross-link HIV spikes, thereby interfering with the assembly of fusion-competent high order complexes. Interestingly, the Fab-bound atomic structure shows interdigitation of indole side chains of two helical peptides that interact through the faces not bound by the antibodies (14). We surmise that these interactions, established after partial desorption from membranes, might provide the structural basis supporting the cross-linking activity.

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