Sphingomyelin and Cholesterol Promote HIV-1 gp41 Pretransmembrane Sequence Surface Aggregation and Membrane Restructuring*

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The interfacial sequence DKWASLWNFNITNWYIK, preceding the transmembrane anchor of gp41 glycoprotein subunit, has been shown to be essential for fusion activity and incorporation into virions. HIV-e, a peptide representing this region, formed lytic pores in liposomes composed of the main lipids occurring in the human immunodeficiency virus, type 1 (HIV-1), envelope, i.e. 1-palmitoyl-2-oleoylphosphatidylcholine (POPC):sphingomyelin (SPM):cholesterol (Chol) (1:1:1 mole ratio), at low (>1:10,000 peptide-to-lipid mole ratio, and promoted the mixing of vesicular lipids at >1:1000 peptide-to-lipid mole ratios. Inclusion of SPM or Chol in POPC membranes had different effects. Whereas SPM sustained pore formation, Chol promoted fusion activity. Even if partitioning into membranes was not affected in the absence of both SPM and Chol, HIV-e had virtually no effect on POPC vesicles. Conditions described to disturb occurrence of lateral separation of phases in these systems reproduced the high peptide-dose requirements for leakage as found in pure POPC vesicles and inhibited fusion. Surface aggregation assays using rhodamine-labeled peptides demonstrated that SPM and Chol promoted HIV-e self-aggregation in membranes. Employing head-group fluorescent phospholipid analogs in planar supported lipid layers, we were able to discern HIV-e clusters associated to ordered domains. Our results support the notion that the pretransmembrane sequence may participate in the clustering of gp41 monomers within the HIV-1 envelope, and in bilayer architecture destabilization at the loci of fusion.

Viral glycoproteins, which are very efficient in attaching virions to target membranes, are thought to catalyze fusion between viral envelopes and target cell membranes through the induction of transient non-lamellar structures at the point where both bilayers would merge, most likely by locally regulating monolayer surface curvatures (1–5). As such, the process is likely to be carried out by high order complexes within confined areas of the interacting bilayers (6, 7). However, it is not clear as yet how glycoprotein-trimer aggregation happens at the bilayer surface to assemble fusion-competent complexes.

Recently, a domain intervening the 624–665 heptad repeat and the 684–706 transmembrane region of human immunodeficiency virus, type 1 (HIV-1) envelope glycoprotein, has been found to participate in the fusion process mediated by this protein (8–11). This region, comprising residues 666–683 of gp160 precursor, is extremely rich in conserved aromatic amino acids. Compelling mutational analysis by Salzwedel and co-workers (8) indicated that this stretch is dispensable for the normal maturation, transport, and receptor binding ability of the glycoprotein, but is required for membrane fusion. These authors suggested, without direct experimental proof, that a possible role for the conserved Trp residues in those processes could involve specific interactions with membrane cholesterol. Further characterization in cell-cell fusion assays revealed three different phenotypes among the studied gp41 mutants: phenotypes showing reduced activity, defective variants unable to mediate fusion, and mutants able to assemble nonexpanding fusion pores (9). Using the hydrophobicity scale of Wimley and White (12), we established that this pretransmembrane (preTM) sequence represents an elongation of the interfacial short segments at the cytoplasmic boundary regions of transmembrane anchors in type 1 integral membrane proteins (10). Moreover, the sequence partitions into membranes adopting an α-helical structure and induces their destabilization, a fact that prompted us to propose the preTM as a second fusion peptide present in the gp41 ectodomain (11, 13).

Given its close proximity to the transmembrane anchor, it is reasonable to assume that the preTM should primarily interact with the HIV-1 envelope. The lipid composition of HIV-1 mem-

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1 The abbreviations and trivial names used are: HIV-1, human immunodeficiency virus, type 1; Chol, cholesterol; IR, infrared spectroscopy; 1, liquid-ordered; 1, liquid-disordered; preTM, pretransmembrane; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; SPM, sphingomyelin; Tm, melting temperature; mN, milineutron(s); cholesterol, α-cholestane-3-an; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)glycine]; LUV, large unilamellar vesicle; N-NBD-PE, N-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; FL-DPPE, 1,2-dipalmitoyl-sn-glycero-phosphatidylethanolamine fluorescent; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid sodium salt; DPX, p-xylene-bis(pyridinium)bromide.
brane as compared with that of host cell plasma membranes has been analyzed by Aloia and co-workers (14, 15). These authors found increased cholesterol-to-phospholipid molar ratios and high levels of sphingomyelin, ~2–3 times that of the host cell surface membranes. The HIV-1 membrane is therefore enriched in cholesterol and sphingomyelin, two lipids that have been related to the occurrence of laterally segregated lipid domains or “rafts” (for reviews, see Refs. 16–18). These findings suggested that virions were probably selective in specific segregated membrane regions through which they would emerge during viral maturation, a fact that has recently received experimental support by Nguyen and Hildreth (19).

Lateral segregation of phases has been indeed visualized in both planar supported lipid layers and in giant unilamellar vesicles, formed from equimolar mixtures of phospholipid-cholesterol-sphingomyelin (20). In this system “liquid-ordered” (l_0) and “liquid-disordered” (l_0) fluid phases were found to coexist. In addition, it has been proposed that cholesterol stabilizes the coexistence of segregated phases when mixed with sphingomyelin (18, 21, 22). Recent experimental spectroscopic and microscopic determinations provide additional support for this suggestion (23, 24). In the present work we investigate the effect of cholesterol (Chol) and sphingomyelin (SPM), two major envelope lipids, on the interaction of gp41 preTM with membranes.

We show that, under conditions required for gel-phase formation, sphingomyelin promotes irreversibility of the peptide aggregation at the membrane surface, thereby stimulating the assembly of discrete lytic units (pores). In addition, in the presence of cholesterol, the peptide promoted intervesicular mixing of lipids, a phenomenon not observed for other sterol analogs. None of these lipids appreciably altered either the main secondary structure attained by HIVc in the membrane or its insertion into lipid monolayers. Using rhodamine-labeled peptide, we observed a good correlation between surface aggregation and activation of bilayer perturbations in the different lipid mixtures. Moreover, epifluorescence of planar supported phospholipid layers containing labeled peptide revealed a preferential association of the gp41 preTM clusters with ordered lipid domains. This lateral segregation might be relevant in HIV-1 fusion.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cholesterol (Chol), POPC, sphingomyelin (SPM), and the fluorescent probes, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (N-NBD-PE) and N-(lissamine rhodamine B sulfo- nyl)phosphatidylethanolamine (N-Rh-PE) were purchased from Avanti Polar Lipids (Birmingham, AL), 1,2-Dipalmitoyl-sn-glycerophosphoethanolamine fluorescence (FL-DPPE), 8-aminoethanolamine-1,3,6-trisulfonic acid sodium salt (ANTS), and 9-decyl ether, and Triton X-100 were obtained from Sigma. The latter fluorescence value did not show dependence on the lipid composition, indicating a similar degree of peptide solubilization in all tested lipid mixtures. The percentage of quenching was estimated from the following expression.

\[
\text{% quenching} = 100 \left( \frac{F_{100} - F_0}{F_{100}} \right)
\]

\[F_0\] and \[F_{100}\] are fluorescence intensities of samples in presence and absence of peptide, respectively, and \[F_{100}\] corresponds to intensity in Triton X-100-solubilized samples. Occasionally, maximal dequenching measurements were carried out in SDS-solubilized samples, always with good correlation. The peak fluorescence intensity of the labeled vesicles and the 100% value 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 fluorescent probes, [4,6-diamidino-2-phenylindole] (DAPI) was used to monitor the location of the peptide within the bilayer, and aggregate. When an aggregate within a membrane has reached a critical size, i.e. it consists of \[M\] peptides, a pore can be created within the membrane, and leakage of encapsulated molecules can occur. It is assumed that the process of peptide binding is random and once a pore has been formed in a vesicle, its contents will leak quickly. Thus, this leakage must be characterized by an all or none mechanism, i.e. the population of vesicles consists of those that did not leak at all and those that leaked all of their contents. Furthermore, the leakage must terminate after a certain period to yield final extents, which depend on peptide-to-lipid ratios. The rate and extent of leakage are assumed to be limited by the rate and extent of formation of surface aggregates of \[M\] or more peptides. The number \[M\] and geometrical considerations dictate the upper size of leaking molecules (32). In most of the cases, the surface aggregation of the peptides is not irreversible data acquisition. Corrected spectra were recorded in a PerkinElmer LS50-B spectrophotometer 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 as described in Ref. 28. The 495-nm excitation was used to excite the N-acetyl-l-tryptophanamide, which does not partition into membranes. The apparent mole fraction partition coefficients, \(K_p\), were determined fitting the experimental values to a hyperbolic function.

\[
F_0/F = 1 + \left( \frac{[L]}{K + [L]} \right)
\]

\([L]\) is the lipid concentration, and \(K\) is the lipid concentration at which the bound peptide fraction is 0.5. Therefore, \(K_p = [W]/K\), where \([W]\) is the molar concentration of water.

Release of vesicular contents to the medium was monitored using the ANTS/DPX assay (29). LUVs 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 20 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 leakage corresponding to the fluorescence of the vesicles at time 0; 100% leakage was the fluorescence value obtained after addition of Triton X-100 (0.5%, v/v).

Membrane lipid mixing was monitored using the resonance energy transfer assay, based on the question as described in Ref. 30. The assay is based on the dilution of N-NBD-PE and N-Rh-PE. Dilution of membrane mixing results in an increased N-NBD-PE fluorescence. Vesicles containing 0.6 mol % of each probe were mixed with unlabeled vesicles at 1:4 ratio (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 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 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.

Surface clustering of HIVc associated to vesicles was monitored following the self-quenching effect as produced in aggregates of Rhodamine labeled peptide (31). Changes in fluorescence intensity were measured at 581 nm (5-nm slit) with excitation set at 550 nm (5-nm slit) on a PerkinElmer LS-50B spectrophotometer. Maximal dequenching (or 0% quenching) was inferred from samples solubilized with Triton X-100 (0.5%, v/v). The latter fluorescence value did not show dependence on the lipid composition, indicating a similar degree of peptide solubilization in all tested lipid mixtures. The percentage of quenching was estimated from the following expression.

\[
\text{% quenching} = 100 \left( \frac{F_{blank} - F_0}{F_{blank}} \right)
\]

\(F_0\) and \(F_{blank}\) are fluorescence intensities of samples in presence and absence of peptide, respectively, and \(F_{blank}\) corresponds to intensity in Triton X-100-solubilized samples. Occasionally, maximal dequenching measurements were carried out in SDS-solubilized samples, always with good correlation. The fluorescence intensity of the labeled peptide in detergent-solubilized samples.

**Analysis of Leakage via Pore Formation**—The model assumes that the peptides added into a vesicle suspension bind, become incorporated into the C-domain of the C-terminal 25-amino acid sequence, and aggregate into a mem-

brane to form a transmembrane pore. When the pore becomes unstable within the bilayer, the membrane has reached a critical size, i.e. it consists of \(M\) peptides, a pore can be created within the membrane, and leakage of encapsulated molecules can occur. It is assumed that the process of peptide binding is random and once a pore has been formed in a vesicle, its contents will leak quickly. Thus, this leakage must be characterized by an all or none mechanism, i.e. the population of vesicles consists of those that did not leak at all and those that leaked all of their contents. Furthermore, the leakage must terminate after a certain period to yield final extents, which depend on peptide-to-lipid ratios. The rate and extent of leakage are assumed to be limited by the rate and extent of formation of surface aggregates of \(M\) or more peptides. The number \(M\) and geometrical considerations dictate the upper size of leaking molecules (32). In most of the cases, the surface aggregation of the peptides is not irreversible

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and depends on $K = CD$, in which $C$ and $D$ denote on and off rate constants of surface aggregation (33, 34). In later studies on the peptide GALA (35, 36), the importance of peptide translocation was emphasized and it was suggested that the surface-associated GALA monomers or aggregates are stabilized in bilayers composed of phospholipids containing a cis unsaturation per acyl chain, which reduces transbilayer insertion.

In the current study we focus on simulating the final extents of leakage induced by the peptide HIV. The calculations, which employ the parameters $M$ (pore size) and $K$ (degree of surface reversibility), use as an input the binding of the peptide and size distribution of vesicles (33, 37). The calculations simulate the final extents of leakage as a function of peptide/lipid ratios. (For a review and relationship between leakage and fusion, see Ref. 38.)

Monolayer Penetration—Surface pressure was determined in a fixed-area circular trough ($\mu$Trough S system, Kilborn, Helsinki, Finland). Measurements were carried out at room temperature and under constant stirring. The aqueous phase consisted of 1 ml of 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 ($\pi_0$) 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, peptide alone induced negligible increase in surface pressure at the air-water interface.

Peptide-Labelled Phospholipid Layers—Phospholipid monolayers were spread from chloroform/methanol 3:1 (v/v) solutions onto a 5 mm sodium sulphate (pH 7.4), 150 mM NaCl subphase, in a thermostated Langmuir-Blodgett trough (NIMA Technologies, Coventry, United Kingdom) as previously described (39, 40). After 10 min to allow for solvent evaporation, monolayers were compressed at 25 cm$^2$/min up to 32 mN/m and then transferred onto a glass coverslip at 5 mm/min.

Epi-fluorescence microscopy observation of the planar supported monolayer was performed with a Zeiss Axioplan II fluorescence microscope. Specific labeling of fluid disordered phase was attained by including 32 mN/m and then transferred onto a glass coverslip at 5 mm/min.

Non-specific labeling at the air-water interface. Peptide was injected into the subphase with a Hamilton microsyringe. At the concentrations used, peptide alone induced negligible increase in surface pressure at the air-water interface.

RESULTS
gp41 PreTM Sequence—Fig. 1 displays the 638–706 sequence of the HIV-1 gp160 precursor, which starts at the C-terminal side of the second helical domain and precedes the intracytoplasmic domain of gp41. The sequence contains two hydrophobic regions: the pretransmembrane (preTM) 664–683 sequence (indicated in bold characters), rich in aromatic residues, and whose average interfacial hydrophobicity (plot below) is therefore high according to the Wimley-White scale (12), and the transmembrane anchor (TM) 706–708 sequence, whose first limiting step is peptide partitioning from the aqueous into the membrane phase. We caution that in the following, when we compare the stability of vesicles of various compositions (Figs. 2–4 and Table II), we have expressed the degree of observed destabilization as a function of actual peptide concentration in the membrane.

In Fig. 2 we show the ability of HIVc to induce leakage of contents in vesicles made of POPC, as compared with that induced in liposomes composed of POPC/SM:Chol (1:1:1 mole ratio), a mixture containing the main lipids present in the viral envelope. The peptide permeabilized POPC vesicles at membrane loads higher than 1:100 peptide-to-lipid mole ratios. In contrast, HIVc-induced permeabilization in POPC/SM:Chol (1:1:1) vesicles started at peptide-to-lipid mole ratios > 1:10,000, i.e. at ~100 times lower membrane loads. Experimental determination of the apparent mole fraction partition coefficients by titrating HIVc in solution with increasing amounts of LUVs (data not shown), rendered in both instances $K_C$ values of ~10$^{-3}$, indicating that, under our experimental conditions, association of the peptide with both types of vesicles was almost quantitative (>95% of added peptide). Thus, in these systems, differ-
aggregation. In this case all experimental points could be fitted quite well to the model ($R^2 = 0.95$). Thus, pore formation was easier in the latter system as compared with pure POPC, and the reduction in surface-aggregation reversibility seemed to be at the origin of this effect.

One important characteristic of the ternary mixture is the presence of SPM/Chol-rich $I_2$ phases that coexist with $I_1$ phases (20, 22–24, 48). Moreover, both Chol and SPM are able to induce phase separation when mixed with PC alone (reviewed in Refs. 16–18). We therefore decided to investigate the effect of these lipids separately. Our aim was also to test whether phase coexistence enhanced peptide-mediated bilayer destabilization in these systems.

In Fig. 3A we show the effect of increasing amounts of SPM on HIV$_c$-induced leakage of liposomes composed of binary POPC:SPM mixtures. It can be observed that the sole presence of SPM in combination with POPC stimulated the lytic activity of the peptide. At a POPC:SPM 1:1 mole ratio, the observed leakage as a function of the peptide dose in the membrane was actually comparable with that detected in the ternary mixture (Fig. 2). In PC:SPM binary mixtures, gel-phase $s_0$ formation starts when SPM reaches 25–30 mol % (22, 49). Data displayed in Fig. 3B demonstrate that the threshold for SPM-induced leakage stimulation in the binary mixtures roughly corresponded to those SPM concentrations. Importantly, as shown in Fig. 3C, leakage induced by the peptide was reduced to the levels measured in pure POPC by heating the samples above the chain melting temperature ($T_m$) of pure SPM ($>37^\circ$C). This inhibitory effect of temperature was not observed with pure POPC in the range of temperatures studied in Fig. 3C (data not shown). Thus, under conditions disrupting the gel-phase SPM-rich domains in the POPC:SPM binary mixture, the leakage process was reduced close to the levels observed in pure POPC. Finally, results displayed in Fig. 3D illustrate the effect of Chol on leakage. This lipid has been described as a $I_1$ phase promoter in binary POPC:Chol mixtures (50). The presence of Chol at molar ratios reported to promote $I_1$ phase formation in POPC did not stimulate leakage by HIV$_c$ as extensively as SPM. To explore the effect of $I_1$ formation in the process, we replaced Chol by sterol analogs unable to induce segregation into domains (51). The latter faculty depends on the planar structure of the ring and the presence of the 3β-hydroxyl group. Therefore we studied the effect of the cholestane, bearing a carboxyl group in position 3, and coprostanol, kinked in between A and B rings, which disturbs its planarity. Although the former compound has been shown to be unable to sustain domain formation, the latter even inhibits it (51). Inclusion of either analog reproduced the membrane peptide-dose requirements observed in the case of pure POPC leakage.

The leakage analysis according to a pore model was subsequently extended to selected examples of the binary mixtures (Table I). The model gave a reasonable fit to the leakage results of POPC:SPM (2:1) vesicles at 25°C ($R^2 = 0.95$). For a pore size of $M = 10$, we obtained $K_s = 0.046$ in this case. Thus, the $K_v$ value computed for the POPC:SPM 2:1 mixture was actually 30 times that obtained in pure POPC. This indicates that the presence of SPM enhanced peptide clustering and subsequent pore formation through the decrease of surface aggregation reversibility. When this mixture was assayed at 50°C, only four points could reasonably be fitted ($R^2 = 0.92$) to the model, which yielded for the same size a lower $K_v = 0.013$. At the highest peptide loads in the membrane, activation of a different mechanism appeared superimposed. In this regard heating in these samples reproduced the leakage process observed in pure POPC (Fig. 2). For POPC-Chol (2:1), we also obtained progressive underestimate of model calculations with increasing pep-
A mediocre fit, $R^2 = 0.83$, was deduced based on 5 points. Here the obtained values were $M = 7$ and $K_s = 0.003$. We may rationalize the underestimate at higher peptide-to-lipid ratios, because there might be also some leakage associated with the fusion process concomitantly taking place at high peptide doses, a phenomenon only detected in Chol-containing vesicles (see below).

A survey of leakage results is shown in Table II. Clearly, the presence of SPM had a stimulatory effect on leakage. By comparison, Chol alone had a moderate enhancing effect on the leakage process. Importantly, peptide partitioning into vesicles was almost complete in all cases. Moreover, as inferred from IR measurements, the secondary structure adopted by HIV, associated to POPC, POPC:SPM (2:1), and POPC:Chol (2:1) vesicles was invariably $\alpha$-helical. The amide I band in these samples always showed a conspicuous peak centered at $1652-1653$ cm$^{-1}$, which is indicative of a preferential helical structure adopted by the peptide fraction bound to vesicles. However, caution must be advised in relation to the SPM-containing samples. SPM contains an amide group displaying a broad IR absorption band whose maximum is located at 1625 cm$^{-1}$ (23, 52). Given the high SPM:HIV mole ratio in these samples (>80:1), SPM absorption overlapped that arising from the peptide. This fact precluded an accurate quantitative analysis of the absorption band components arising from the peptide, and, consequently, only the position of the band maximum is reported.

Penetration into Lipid Monolayers—The leakage effects described appeared to arise in great part from the promotion of HIV clustering in the bilayer under conditions allowing lipid phase coexistence. However, differences in the penetration capacity and/or specific requirement of a lipid for insertion might also be invoked to explain them. Peptide insertion in membranes may be evaluated by means of the lipid monolayer technique (53). HIV insertion into monolayers of varied compositions was assayed by measuring changes in surface pressure at the fixed $\pi_0 = 20.0$ mN m$^{-1}$ (Table II). Comparable changes in surface pressure were observed for pure POPC.
monolayers and the rest of mixtures tested, indicating that the peptide penetrated similarly into them. Moreover, the observed exclusion pressures, \( \pi_{ex} \), (i.e. \( \pi_{0} \) at which \( \Delta \pi = 0 \) after injection of the peptide into the subphase) for POPC, POPC:SPM (2:1), and POPC:Chol (2:1) monolayers (data not shown) were all above lateral pressures postulated to arise from the lipid packing densities existing in biological membranes (\( \pi_{ex} \geq 30 \) mN m\(^{-1}\); Ref. 54). Thus, HIV\(_v\) was able to penetrate efficiently into vesicular membranes of these compositions.

**Intervesicular Lipid Mixing**—Intervesicular lipid mixing induced by HIV\(_v\) was only detected in Chol-containing vesicles (Fig. 4). Panel A displays lipid mixing as a function of the peptide dose in the membrane for the ternary mixture as compared with the binary mixtures and pure POPC. The peptide was approximately an order of magnitude more potent in the ternary mixture than in the binary one, and virtually no fusion signal was detected for POPC and POPC:SPM (2:1). In fact, none of the POPC:SPM binary mixtures used in this study (Fig. 3) supported vesicular lipid mixing induced by the peptide. That means that addition of Chol to POPC:SPM (1:1) induced lipid mixing activity of the peptide at 1:1000 lipid-to-peptide mole ratios in membranes, or that replacing one half of the POPC by SPM in the binary POPC:Chol (2:1) mixture stimulated this process roughly 10 times. The stimulatory effect of Chol on fusion was not reproduced by the cholesterol analogs 5a-cholesten-3-ane and coprostanol (Fig. 4, B and C).

**Surface Aggregation of the Peptide**—Surface aggregation of the peptide was tested using the fluorescently labeled Rho–HIV\(_c\) sequence. When fluorophore molecules are in close proximity, rhodamine emission diminishes (31, 55). Thus, Rho quenching efficiency correlates with the aggregation state of the peptide. Experiments in Fig. 5 describe the effect of the lipid environment on the emission of Rho-HIV\(_v\) associated to LUVs. We note that, under the measuring conditions in this figure, >90% of added peptide was actually associated with vesicles, in all tested lipid compositions. Emission spectra (panel A) obtained in POPC LUVs were consistent with peptide forming aggregates at a peptide-to-lipid ratio of 1:50, but not at 1:1000. When increasing amounts of unlabeled peptide substituted for labeled peptide, Rho dequenching was observed, indicating that indeed the observed fluorescence attenuation was because of aggregate formation.

The percentage of Rho quenching was subsequently used to estimate in different lipid mixtures the degree of aggregation as a function of the peptide-to-lipid ratios (Fig. 5B). Data displayed in this panel reflect that Rho was more quenched at all tested peptide-to-lipid ratios in POPC:SPM:Chol (1:1:1) LUVs than in vesicles of any other composition. The data also reflect that, in Chol-containing mixtures, Rho appeared already quenched by ~50% at the lowest peptide-to-lipid ratios tested. Moreover, POPC:SPM (1:1) and POPC:Chol (2:1) mixtures caused a higher degree of quenching than pure POPC. In summary, the quenching efficiency data as a function of the peptide dose in LUVs suggest that indeed peptide-aggregates are involved in membrane perturbations, and that phase-coexistence promotes HIV\(_v\) clustering.

To get better insight into the latter phenomenon, we next carried out planar supported phospholipid layer experiments (Fig. 6). Using this technique Dietrich et al. (20) visualized lipid phase coexistence in POPC:SPM:Chol lipid mixtures. Accord-

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**Table I**

| Lipid composition         | \( M \) | \( K' \) | \( R^2 \) |
|---------------------------|---------|---------|---------|
| POPC                      | 10      | 0.0017  | 0.91    |
| POPC:SPM:Chol (1:1:1)     | 10      | 0.1     | 0.95    |
| POPC:SPM (2:1)            | 10      | 0.046   | 0.95    |
| POPC:SPM (2:1)\(_x\)      | 10      | 0.013   | 0.92    |
| POPC:Chol (2:1)           | 7       | 0.003   | 0.83    |

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\( ^a \) Leakage was measured at 50 °C.
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**DISCUSSION**

The existence of in-plane lipid heterogeneity has been related to the induction of lateral segregation of proteins (16–18). Other protein-lipid interactions, such as membrane insertion after partitioning from the aqueous phase, may be modulated by lipid phase coexistence as well (see, for instance, Refs. 56 and 57). HIVenvelope, the peptide sequence used in this work, was extremely hydrophobic at interfaces. The theoretically computed $K_a$ values according to Ref. 12 were $\sim 10^6$ and $10^{13}$ for the completely unfolded and folded sequences, respectively.2 Accordingly, in our experiments the presence of lipids promoting in-plane heterogeneity was not required for effective partitioning of the peptide from the aqueous phase into membranes. HIVenvelope did not require either phase coexistence for membrane insertion or folding into a defined secondary structure thereafter. Thus, the effects described here specifically pertain to the clustering processes that, following immersion and folding into bilayers, lead to induction of pore formation and intervesicular mixing of lipids by peptides. We propose that such effects may reflect a physiologically meaningful regulation of the gp41 preTM interaction with membranes.

**TABLE II**

Interactions of HIVenvelope with model membranes: influence of lipid composition on leakage of contents, adopted conformation in LUV, and penetration into lipid monolayers

| Lipid composition | Leakage$^a$ | Amide I$^b$ | $\Delta \sigma$$^c$ |
|-------------------|------------|------------|----------------|
| POPC              | 75:1       | 1653       | 11.7           |
| POPC:Chol (9:1)   | 100:1      | 11.1       |
| POPC:Chol (2:1)   | 150:1      | 11.3       |
| POPC:Chol (1.5:1) | 150:1      | 11.1       |
| POPC:coprostanol (2:1) | 75:1 | 11.0       |
| POPC:cholestane (2:1) | 75:1 | 14.0       |
| POPC:SPM (9:1)    | 125:1      | n.d.       |
| POPC:SPM (4:1)    | 160:1      | n.d.       |
| POPC:SPM (2:1)    | 750:1      | 1652       |
| POPC:SPM (1:1)    | 300:1      | 9.8        |
| POPC:SPM (1:1)  | 2000:1     | 8.2        |
| POPC:SPM:Chol (1:1:1) | 1500:1 | 8.4        |

$^a$ Lipid-to-peptide mole ratios required in the membrane to obtain 50% leakage of vesicular contents.

$^b$ Position of the maximum absorption in the amide I band corresponding to the peptide fraction bound to vesicles, as determined by IR spectroscopy.

$^c$ Increase in surface pressure after peptide (5.5 μM) addition to lipid monolayers at the initial pressure of 20 mN m$^{-1}$. At the concentration, used surface activity of the peptide alone was negligible.

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phase and fluid-disordered regions, as described for other proteins (39). Our planar supported monolayer data shown in Fig. 6 seem to support this latter hypothesis, because peptides appeared homogeneously distributed in disordered phases but accumulated as clusters at the boundaries between demixing phases.

The molecular basis sustaining the clustering process, also required for pore formation, is still unclear. We may speculate that factors such as the optimization of the surface interaction between acyl chain and the peptide might play a role in this process. HIVc monomers interacting with POPC membranes are expected to localize immersed in the membrane interface with the main axis parallel to the plane of the bilayer (58). Recent nuclear magnetic spectroscopy resonance data have added support to this type of HIVc-membrane interaction (59). This surface state has been shown to be more energetically favored in lipids containing less ordered acyl chains that better accommodate the head group displacement because of peptide insertion (36). The local increase of ordered acyl chains in SPM-rich gel-phase domains might favor the inserted state of the peptide, which would increase the probability of subsequent pore formation. At any rate, our results might be best explained according to a model in which peptide clusters show more affinity than monomers for stably existing lateral lipid domains in ordered phases (17). The clustering phenomenon might be restricted to the boundaries between phases (Fig. 6).

Munoz-Barroso et al. (42) found that DP178 could inhibit more readily solute-permissive fusion pores than lipid-permissive fusion pores. In a later study, Kliger and co-workers (43) suggested that this dissimilar effect might be caused by secondary binding of DP178 to the membrane-bound preTM region, thereby interfering with further oligomerization of gp41. Our data are consistent with this supplementary mode of action of DP178, in that they support the involvement of the preTM region in gp41 clustering.

Cholesterol Effects—Our data indicate that Chol induced a
subtle permeability increase, although this effect was modest in comparison with that induced by SPM (Table II) and seemed to correlate with the promotion of lipid mixing. The leakage phenomenon in this system could not be optimally described on the basis of a pore model (Table I). It should be noted that cholesterol has been reported to interfere with pore formation. In the case of the amphipathic peptide GALA, the inclusion of cholesterol in POPC vesicles resulted in reduced efficiency of pore formation (34). Numerous other cases are reviewed in the latter article, where inclusion of cholesterol in membranes reduced leakage induced by peptides.

HIV, induced the type of bilayer perturbations required for fusion only in vesicles containing Chol (Fig. 4). The presence of Chol seems to be a specific requirement for HIV-1 infection (60–62). According to Mateo and co-workers (50), in POPC:Chol (2:1) LUVs, \( l_\alpha \) and \( l_\beta \) phases also coexisted under our experimental conditions. Sterol analogs not supporting \( l_\alpha \) phase formation (51) interfered with the capacity of HIV, to induce intervesicular mixing of lipids. However, it cannot be ruled out that the Chol requirement for fusion might be because of factors other than phase coexistence induced by this lipid. One obvious possibility is that Chol effect might stem from its ability to promote non-lamellar configurations of the bilayer, a characteristic directly related to induction of fusion (63). Nevertheless, this Chol trait would not satisfactorily explain the fusion stimulation observed when half of POPC was replaced by SPM (a lamellar-type lipid that strongly inhibits non-lamellar phase formation).

It seems more likely that, in the presence of Chol, HIV, perturbed bilayers from an interfacial location analogous to that present in pure POPC membranes, and that this compound promoted peptide clustering therein (Fig. 5). The nature of these perturbing peptide complexes remains to be determined, but we may anticipate that peptides in POPC and POPC-Chol share a main \( \alpha \)-helical secondary structure. The surface aggregation data obtained in the ternary mixture, as compared with the binary ones (Fig. 5), are consistent with a synergistic effect, according to which peptide clustering appears to be facilitated in coexisting lipid phases that contain Chol. The presence of Chol stimulates ordered phase formation in phospholipid/spingolipid mixtures (22, 48).

**Functional Implications—**Membrane microdomains enriched in spingolipids and cholesterol, or “rafts,” have been postulated to be functionally important for the HIV-1 infectious cycle (reviewed in Ref. 64). The results in this work suggest some functional consequences of the high levels of these lipids detected in the HIV-1 envelope. Most importantly, they point to the existence of lipid domains within these membranes as an important regulatory factor of viral fusion. Our data confirm that the interfacial gp41 preTM sequence behaves as a signal for lipid domain targeting. The existence of a long interfacial preTM sequence has been postulated to represent a common structural motif present in fusion glycoproteins of several virus families (10). It remains to be determined whether these viral sequences share the capacity to act as lipid domain sensors, and whether they further exploit this lipid recognition to induce destabilization of the bilayer architecture during the fusion event (see below).

The experimental data reported here suggest various mechanisms through which lipid domain coexistence might regulate the HIV-1 gp41 fusion reaction. The high SPM/Chol content in the HIV-1 envelope might sustain clustering of gp41 preTM sequences and further activation of their fusogenic action. Formation of specific high order complexes has been postulated to represent an important step in gp41-induced membrane fusion (42, 43). Several trimers are probably required for a fusion pore to form, indicating that lateral aggregation of gp41 trimers must be a prerequisite for fusion. We speculate that lipid domain targeting by the gp41 preTM might cause surface aggregation of gp41 trimers, thereby assisting in the formation of the oligomeric complexes competent in fusion pore opening (1, 6).

In support of this hypothesis, it must be mentioned that the functionally characterized gp41 preTM mutants showed impaired fusion activity or formation of fusion pores unable to expand (9).

Our results on HIV, clustering also suggest that gp41-induced fusion pores might open within or in the vicinity of demixing phase boundaries. Recent observations indicate that the HIV-1 fusion process is initiated by the destabilization of gp41-containing membranes (65). The experiments in this work demonstrate that the gp41 preTM has the capacity to compromise envelope-like bilayer integrity, and that this phenomenon may be regulated by the presence of lipid domains. Simultaneous insertion of preTM sequences in Chol/SPM-rich membrane patches, delimited by the gp41 complexes, might induce the formation of highly curved membrane structures or local protrusions (3). It has been argued that creation of membrane projections, dimples (3), or more recently, nipples (5), by viral fusion proteins represents the main energetic barrier for initial bilayer merging and subsequent fusion pore formation. In addition we speculate that, in gp41-mediated fusion, preTM-induced nipples might be enriched in Chol/SPM. Taking into consideration the model for membrane fusion recently proposed by Kuzmin and co-workers (5), we suggest two mechanisms by which raft-type lipids might assist in the formation of the lipidic structural intermediates leading to the opening of the fusion pore: 1) enhanced adherence at the tips of the interacting nipples, which would facilitate contacting of cis monolayers, and 2) in plane heterogeneity that might facilitate tilting of lipids in trans-contacting monolayers, thereby lowering the free energy of fusion pores.

**Concluding Remarks**—Soon after the identification of HIV-1 as the etiologic agent of acquired immunodeficiency syndrome, it was recognized that Chol depletion from the lipid envelope could cause loss of viral infectivity (60, 61). In addition, early observations by Aloia and co-workers (14, 15) indicated that fluidization of the viral envelope also leads to viral inactivation. The identification of the gp41 preTM as a lipid domain targeting sequence provides a mechanistic basis for understanding, at the molecular level, the HIV-1 infectivity loss caused by sterol depletion and/or fluidization.

Gp41 active sequences have gained much attention as targets for specific antiviral therapeutics (66, 67). As discussed by Zwick et al. (44), the highly conserved preTM region is accessible to broadly neutralizing antibodies. As such it constitutes a potential target for drug and vaccine design. However, a better structural knowledge on this region is required to elicit an effective immune response using epitope-targeted immunogens. Our experimental work gives support to the notion that gp41 preTM may establish specific interactions with Chol-containing membranes, i.e. this lipid plays an important role at regulating the structural state of gp41 preTM sequence. This knowledge might help in the design of preTM-based immunogens as vaccine candidates, as well as in the development of new inhibitory drugs.

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