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Lysophosphatidylcholine mediates the mode of insertion of the NH_2-terminal SIV fusion peptide into the lipid bilayer

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We report here on the interaction of a synthetic 12 residue peptide corresponding to the N-terminal sequence of gp32 from SIV with phospholipid bilayers. This peptide has been shown to induce lipid mixing of PC/PE/SM/Chol LUV (large unilamellar vesicles) at pH 7.4 and 37°C [(1992) in: Advances in Membrane Fluidity, vol. 6, pp. 365–376, Wiley-Liss]. In the present study, this fusion process was inhibited by the addition of lysophosphatidylcholine (lysoPC) to the lipid bilayer of PC/PE/SM/Chol LUV. Fourier transform infrared spectroscopy (FTIR) reveals that the orientation of the SIV fusion peptide with respect to the lipid acyl chains depends on the presence of lysoPC in the lipid bilayer but that the peptide secondary structure and the amount of lipid-associated peptides do not depend on the lipid composition. The peptide is obliquely inserted into the lipid bilayer of vesicles without lysoPC, whereas it is oriented parallel to the lipid–water interface in the vesicles containing lysoPC. The data provide evidence that the orientation of the SIV fusion peptide depends on the lipid composition, and that this mediates its fusogenic activity.

SIV fusion peptide; lysoPC; Lipid mixing; FTIR spectroscopy

1. INTRODUCTION

Although a great deal of effort has been made to understand the genetic and immunological aspects of HIV infection, the molecular mechanism of fusion between the virus and its host cell remains poorly understood. Most viral fusogenic proteins contain a short N-terminal hydrophobic segment which has been proposed to interact with the lipid membrane during the fusion event [1,2]. Mutagenesis studies have confirmed that modifications which disrupt the distribution of the hydrophobic amino acids in the N-terminus of influenza hemagglutinin [3], gp41 of HIV [4,5] and gp32 of SIV [6] inhibit syncytium formation without affecting glycoprotein synthesis and processing or receptor binding, suggesting that hydrophobicity plays a key role in the fusion process.

Chemically synthesized peptides corresponding to the N-terminal segment of influenza virus hemagglutinin (HA2) [7–10], SIV gp32 [11,12] and HIV gp41 [13,14] interact with lipid model membranes as α-helical structures [7,11,14] and mediate the fusion of lipid vesicles. The functional role of these fusogenic peptides was confirmed by the finding of a correlation between their fusogenic activity in model systems and their in vivo activity [3,13]. Recent data suggest, however, that the hydrophobicity of the N-terminal fusion peptide is not the only parameter required for fusion to occur, and that the orientation and the secondary structure of the peptide at the lipid–water interface play a crucial role during the fusion event [15,16]. Burger et al. [17] found that while the Influenza wild-type peptide penetrates a dioleoyl phosphatidylcholine/dioleoyl phosphatidylethanolamine/cholesterol monolayer more effectively than a modified peptide, both peptides have similar α-helical content, suggesting that the formation of an α-helix is not sufficient for fusion activity. Computer analysis predicted that the N-terminal fusogenic domains of a series of enveloped viral proteins were oriented obliquely with respect to the lipid–water interface, thereby favoring a local membrane destabilization and generating new lipid arrangements which are thought to be associated with the initial steps of membrane fusion [18]. The phase preference of lipids has been related to the average shape of the molecules. Bilayer-preferring lipids are thought to adopt an overall cylindrical shape. In contrast, lipids which readily form the inverted hexagonal (H_{II}) phase are thought to be conical with the polar headgroup at the smaller end of the cone. An example of such a lipid is phosphatidylethanolamine (PE). Lipids such as lysophosphatidylcholine (lysoPC), with a relatively large hydrophilic moiety, prefer micellar organizations in excess water. The shape–structure relationship also applies to mixed lipid systems, such as a mixture of equimolar amounts of lysoPC and PE, which organizes in lamellar structures [19]. Further-

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more, it should be noted that the concept, besides geometrical dimensions, takes into account dynamic properties of the lipids, as well as headgroup hydratation and inter- and intramolecular interactions. Transition between the bilayer structure and inverted phases can be induced by changes in temperature, pH, ionic strength and hydration. The phase change can be induced by the addition of divalent cations or polypeptides, for example antiviral activity of structurally different compounds has been associated to their capacity of increasing the bilayer/non-lamellar lipid structure transition temperature [20–22].

We have reported previously that a synthetic peptide corresponding to the 12 amino acids of the N-terminus of gp32 of SIV induces fusion of large unilamellar vesicles, provided the liposomes contain PE [11,14]. In this report, we have investigated the role of the lipids in the fusion process induced by the SIV N-terminal 12 residue peptide. Lipid mixing of large unilamellar vesicles depends on the lipid composition and specifically on the lysoPC content. We have also studied the structure and the orientation of the peptide associated to LUV in the absence and the presence of lysoPC. The orientation of the peptide in the lipid bilayer determines the observed fusogenic activity.

2. MATERIALS AND METHODS

2.1. *Materials*

Egg PE, egg phosphatidylcholine (PC), cholesterol (Chol), sphingomyelin (SM), and lysoPC were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

N-(Nitrobenzo-2-oxa1,3-diazol) phosphatidylethanolamine (NBD-PE) and *N*-(lissamine rhodamine B sulfonyl) phosphatidylethanolamine (Rh-PE) were from Avanti Polar Lipids Inc. (Birmingham, AL, USA).

2.2. *Peptide synthesis*

Peptide was synthesized by solid-phase synthesis using a commercially available peptide synthesizer (model Biolynx; Pharmacia Biochrome, Cambridge, UK) and the pre-weighed Fmoc amino acid OPFP esters (Pharmacia Biochrome, Cambridge, UK). Acylation rate was monitored by the Bioplus software by measuring the release of an anionic dye (Acid violet, 17.3 mg/100 ml dimethylformamide and 0.14 ml diisopropylethylamine) at 600 nm. The peptides were cleaved from the resin using trifluoroacetic acid containing 2% anisol and 2% ethanedithiol for 2 h followed by ether precipitation. Each peptide was purified to more than 95% purity by HPLC on a TSK 120T reverse-phase column (7.5 × 300 mm) (Pharmacia, Sweden). The peptides typically eluded at 65% acetonitrile (between 65 and 75%) using a linear gradient over 90 min from 0 to 80% acetonitrile in 0.1% trifluoroacetic acid. The sequence was verified by protein sequencing on an Applied Biosystem sequencer. The peptide was dissolved in TFA, and after evaporation of the solvent, DMSO was added to give a final peptide concentration of 8×10^{-4} M. The stock solutions were stored at 0°C.

2.3. *Vesicles preparation*

Multilamellar vesicles (MLV) were obtained by vortexing a lipid film in a Tris buffer (10 mM Tris, 150 mM NaCl, 0.1 mM EDTA, 0.02% NaN_3, pH 7.4).

Large unilamellar vesicles (LUV) were prepared according to the extrusion procedure of Hope et al. [23] using an Extruder (Lipex Biomembranes Inc., Vancouver, Canada). Briefly, frozen and thawed MLV were extruded 10 times through two stacked polycarbonate membranes with a pore size of 0.1 μm (Nucleopore Corp., Pleasanton, CA, USA).

2.4. *Attenuated total reflection fourier-transformed infrared spectroscopy (ATR-FTIR)*

A complete description of the procedures allowing the determination of the peptide secondary structure and orientation is given in Goormaghtigh et al. [24,25] and in Martin et al. [14]. ATR-FTIR spectra were recorded at room temperature on a Perkin-Elmer 1720X FTIR spectrophotometer equipped with a liquid nitrogen-cooled mercury cadmium telluride (MCT) detector at a nominal resolution of 4 cm^{-1}, encoded every 1 cm^{-1}. The spectrophotometer was continuously purged with air dried on a silica gel column (5 × 130 cm). The internal reflection element was a germanium plate (50 × 50 × 2 mm, Harrick EJ2121) with an aperature angle of 45°, yielding 25 internal reflections. For each spectrum, 128 scan cycles were averaged; in each cycle the sample spectra were ratioed against the background spectra of a clean germanium plate, using a shuttle to move the sample or reference into the beam. For polarization experiments, a Perkin-Elmer gold wire grid polarizer was positioned before the sample and the reference.

2.4.1. *Sample preparation*

SIV peptides dissolved in DMSO at a final concentration of 1 mg/ml, were added to liposomes at a molar lipid/peptide ratio of 300/1 (2.9×10^{-6} M lipid and 10^{-8} M peptide in a total volume of 200 μl). After overnight incubation at 37°C, the lipid–peptide complex was separated from the free peptides by filtration of the sample through an anisotropic hydrophilic YMT ultrafiltration membrane (cut off 30,000 Da) of a Centrifree Micropartition System (Amicon). The driving force for ultrafiltration is provided by centrifugation at 2,000 × *g* on a fixed-angle rotor of a Sorvall superspeed RC2-B centrifuge. Oriented multilayers were obtained by slow evaporation of the liposomes under N_2 stream and at room temperature [26] on one side of the germanium plate. To differentiate between the α-helix and the random structures, the multilayers were exposed 3 h to N_2 saturated with D_2O [27].

2.5. *Lipid mixing assay*

Lipid mixing was determined by measuring the fluorescence intensity change resulting from the fluorescence energy transfer between the probes NBD-PE and Rh-PE, as described by Struck et al. [28]. Fluorescence was monitored using a SLM 8000 spectrofluorimeter with excitation and emission slits of 4 nm. Both probes were added to the lipid film and LUV were prepared as described above.

Liposomes containing both probes at 0.6% (molar ratio) each, were mixed in a 1/9 mole ratio with probe free liposomes at a final lipid concentration of 3×10^{-4} M. The initial fluorescence of the 1/9 (labeled/unlabeled) suspension was taken as 0% fluorescence and the 100% fluorescence was determined using an equivalent concentration of vesicles prepared with 0.06% of each fluorescent phospholipid. The suspensions were excited at 470 nm and the NBD fluorescence was recorded at 530 nm.

3. RESULTS

3.1. *Lipid mixing*

Lipid mixing of LUV was monitored by resonance energy transfer between NBD-PE and Rh-PE incorporated into the liposome membrane [28]. Since small unilamellar vesicles may have phospholipid packing defects that render them more susceptible to fusion [29,30], we have investigated the fusion activity of SIV peptide using large unilamellar vesicles.

Addition of the SIV peptide to LUV of PC/PE/SM/chol (1:1:1:1.5 molar ratio) induces a lipid mixing between labeled and unlabeled vesicles (Fig. 1).

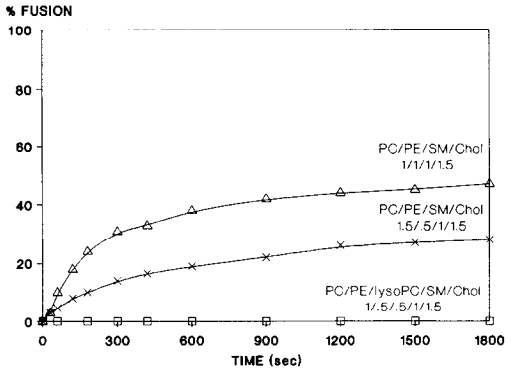


Fig. 1. Fusion of large unilamellar vesicles (LUV) induced by the SIV12 aa peptide at various lipid compositions (the represented curve is an average of five independent experiments, and the error on the fusion percentage is 2%). Labeled and unlabeled vesicles were mixed at a 1/9 ratio. At time 0, the peptide in DMSO was added and the increase in fluorescence, due to a decrease in fluorescence energy transfer following liposome–liposome fusion was monitored at 530 nm, pH 7.4 and 37°C. DMSO up to 2% (v/v), which is the maximal concentration used, did not modify the fluorescence. The experimental conditions were the same for all the lipid compositions tested. The total lipid concentration was 3×10^{-4} M and the peptide concentration 1.3×10^{-5} M. The molar lipid/peptide ratio is 25.

PE has been shown to form non-lamellar lipid structures [31] and to enhance the fusion process when it is inserted in the lipid bilayer. In order to define the role of PE in the fusion process better, a fraction of PE was replaced with lysoPC. Fusion did not occur after addition of SIV12aa to LUV of PC/lysoPC/PE/SM/Chol (1:0.5:0.5:1:1.5 molar ratio). This inhibition was not the consequence of an insufficient amount of PE, since SIV12aa triggers lipid mixing of PC/PE/SM/Chol LUV (1.5:0.5:1:1.5 molar ratio) (Fig. 1) when lysoPC was replaced with a corresponding amount of PC. The inhibition effect observed could be interpreted in terms of molecular 'shape': lysoPC adopts a conical shape complementary to that of PE [32], and in an equimolar mixture of lysoPC and unsaturated PE, lysoPC stabilizes the bilayer organization [19]. Moreover, Fig. 1 suggests that the overall PC-to-PE ratio modulates the fusogenic activity: when the PC/PE molar ratio increases, the fusogenic activity of SIVWT decreases.

Table I

Proportion of the different secondary structures of SIV12aa in the absence and in the presence of lipid

SIVWT	α-Helix (%)	β-Sheet (%)	Random (%)	β-Turn (%)
+ DMSO	0	67 ± 5	33 ± 5	0
+ PC/PE/SM/Chol	35 ± 5	23 ± 4	35 ± 5	7 ± 2
+ PC/PE/lysoPC/ SM/Chol	30 ± 5	20 ± 4	35 ± 5	15 ± 5

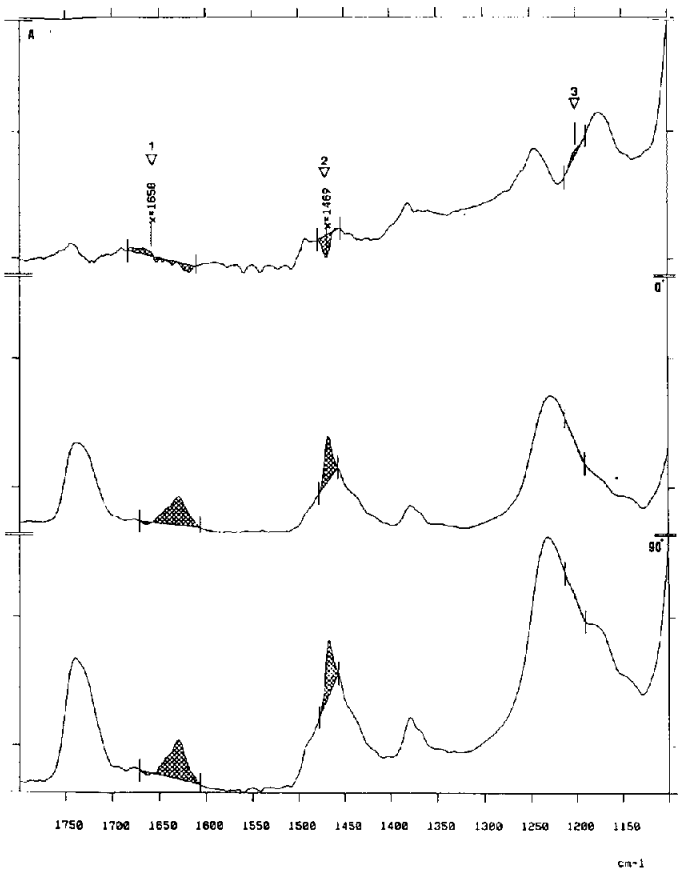
The molar lipid/peptide ratio is 200. The percentage of structure is the average of three independent measurements.

3.2. *Conformational studies*

The FTIR spectra of SIV12aa peptide dissolved in DMSO reveal a high content of β-sheet structures (Table I). After incubation with LUV of PC/PE/SM/Chol (1:1:1:1.5 molar ratio) or LUV of PC/lysoPC/PE/SM/Chol (1:0.5:0.5:1:1.5 molar ratio) at a lipid/peptide molar ratio of 200, overnight at 37°C, the proteoliposomes were separated from free peptide by centrifugation on a centrifree system (cut off 30 kDa). In contrast to lipid-free peptide, the lipid-associated peptide displays some α-helical structure (Table I) characterized by the appearance of a new large peak centered around 1,650 cm^{-1}. This significant increase of the α-helix (± 30%) content is accompanied by a decrease of the β-sheet content (Table I). The structural changes observed for LUV of PC/PE/SM/Chol and LUV of PC/PE/LysoPC/SM/Chol are identical (Table I).

Since the amount of SIV peptide associated with the lipid is difficult to evaluate by colorimetric methods, because of the small amount of an hydrophobic peptide and the presence of lipids, the peptide/lipid ratio was determined by evaluating the $S_{amide}/S_{\nu(C=0)lipid}$ ratio on the IR spectra (S_{amide} is the area of amide I measured between 1,680 cm^{-1} and 1,600 cm^{-1}, and $S_{\nu(C=0)lipid}$ is the area of the lipid ν(C=0) band between 1,770 cm^{-1} and 1,700 cm^{-1}) [24]. In the two systems (with and without lysoPC), the ratios $S_{amide}/S_{\nu(C=0)lipid}$ were identical for equal lipid concentrations, suggesting that the same amount of peptide is associated to the vesicles in the presence and in the absence of lysoPC in the lipid bilayer.

The spectra of SIV12aa inserted into LUV of PC/PE/SM/Chol (1:1:1:1.5 molar ratio) and LUV of PC/lysoPC/PE/SM/Chol (1:0.5:0.5:1:1.5 molar ratio) were recorded with parallel (0°) and perpendicular (90°) polarized light. The difference spectra 90°–0° (Fig. 2) show a negative deviation around 1,657 cm^{-1} for the helical structure associated with the LUV of PC/lysoPC/PE/SM/Chol which characterizes an orientation of the helix axis parallel to the ATR element surface. No deviation is observed for the α-helix associated to the fused LUV of PC/PE/SM/Chol, indicating an oblique orientation (Fig. 2). The curve fitting applied to the polarized spectra in the amide I region allows for the evaluation of the dichroic ratio for the α-helical structure and the calculation of a corresponding angle between the long axis of the α-helix and a normal to the germanium plate. For the helical structure associated to the LUV of PC/lysoPC/PE/SM/Chol the angle is equal to 90 ± 5° and to 5 ± 5° for LUV of PC/PE/SM/Chol. To determine these orientations, a 27° deviation angle between the α-helix axis and the C=O transition dipole moment described by Rothschild et al. [33] was taken into account by introducing an order parameter $S_{C=O} = (3\cos 227° - 1)/2$ so that $S_{helix} = S_{measured}/S_{C=O}$ [22,27]. The angle between the helix axis and the perpendicular to the bilayer we arrive at is therefore a minimum esti-


A
1
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2
x=1469
3
0°
90°
1750 1700 1650 1600 1550 1500 1450 1400 1350 1300 1250 1200 1150
cm-1

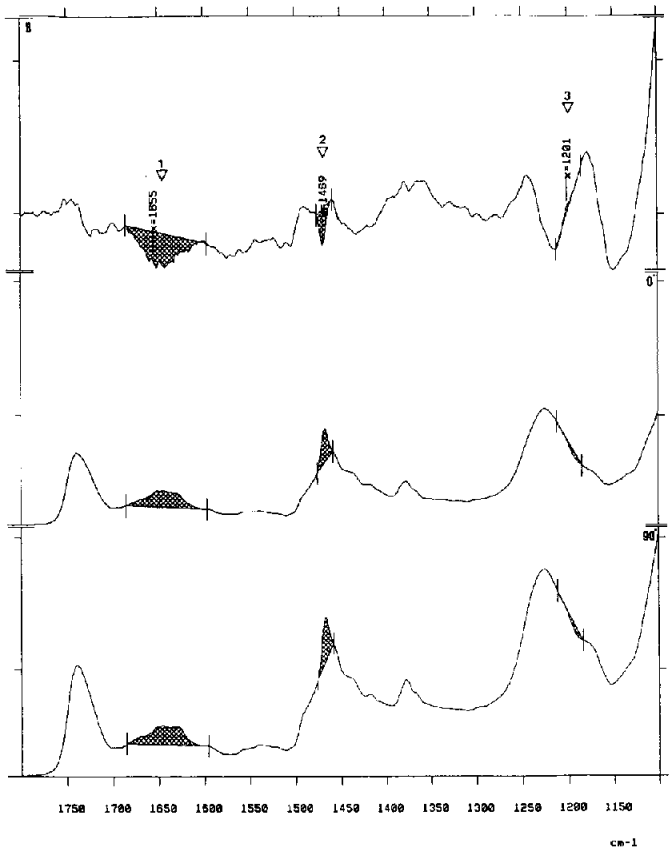
B
1
x=1655
2
x=1489
3
x=1201
0°
90°
1750 1700 1650 1600 1550 1500 1450 1400 1350 1300 1250 1200 1150
cm-1

Fig. 2. ATR spectra of SIV12AA inserted in LUV PC/PE/SM/Chol (A) and LUV PC/PE/lysoPC/SM/Chol (B), recorded with the 90° and 0° polarization. The dichroism spectrum obtained by subtracting the (90°–0°) recorded spectra is plotted on the top of the figure, expanded 3-fold in the ordinate direction. The arrows indicate the protein amide I (1), the phospholipid $\delta(CH_2)$ at 1,468 cm^{-1} (2) and the $\gamma\delta(CH_2)$ at 1,200 cm^{-1} (3). ←

mate, and an orientation of the helix axis closer to this perpendicular would result from considering other sources of disorder, such as an imperfect parallelism between the bilayer and the germanium crystal surface. Dichroic ratios corresponding to the β-sheet structure revealed no significant orientation.

4. DISCUSSION

It is difficult to imagine that the lipid bilayer structure is preserved during the fusion process. PE is known to form non-lamellar lipid structures. A relationship between non-lamellar lipid structure formation (H_{II}) and the fusion process has been proposed [34]. A number of structurally different compounds (cholesterol sulfate [21], carbobenzoxy-D-Phe-L-Phe-Gly [20], tromantadine [35]) are bilayer stabilizers and show antiviral activity. Some of these agents inhibit both model membrane as well as viral fusion, suggesting that there is a relationship between the effect of these drugs on the lipid structure and their antiviral activity. Their antiviral activity results from a change in the biophysical properties of the lipid membrane which is correlated with an increase of the bilayer/non-lamellar transition temperature [36].

If lipid polymorphism is important in biological processes, such as fusion, and if in these processes modulation of lipid structure is triggered by lipid–protein interactions, the important question arises as to whether proteins will be able to induce changes in lipid phase behavior upon entering the membrane through an aqueous phase. Until now, only the measles virus fusion peptide has been shown to increase isotropic ^{31}P resonance formation, but the peptide apparently produced only a modest increase in the rate of fusion. However there is a lack of quantitative correlation between peptide-induced isotropic ^{31}P resonance formation and the rates of peptide induced viral fusion [37]. This peptide also has a modest activity in lowering the bilayer to the hexagonal transition temperature of dielaidoylphosphatidylethanolamine [38]. However, this peptide was found to be difficult to complex with phospholipids because of its insolubility in a variety of both polar and non polar solvents. The SIV12aa fusion peptide was shown to lower the hexagonal transition temperature of palmitoleoylphosphatidylethanolamine (DPOPE). The pure lipid has a hexagonal transition temperature (T_h) of 43°C. The SIV fusion peptide lowered the T_h by 290°/mol fraction. A good linearity of the shift in T_h with concentration was observed (to be published).

We attempted to correlate the fusogenic activity of the SIV12aa peptide with its mode of insertion into the lipid bilayer. Analysis of the polarized spectra provides evidence that the orientation of the α-helix axis depends on the presence of lysoPC in the lipid bilayer. Without lysoPC in the vesicles membrane, the α-helix axis is oriented obliquely in the lipid membrane, whereas in the presence of lysoPC the α-helix is parallel to the lipid–water interface, suggesting its non-insertion into the membrane. The data reported here suggest that the membrane insertion of the SIV fusion peptide plays an important role in vesicle fusion and in virus–cell membrane fusion. It has been proposed that the fusion process involves the penetration of the fusion peptide into the target membrane favoring inverted phase formation. The fact that the 12aa SIV fusion peptide lowers the transition temperature of DPOPE from a bilayer to an hexagonal phase is in agreement with this proposal.

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