Permeabilization and Fusion of Uncharged Lipid Vesicles Induced by the HIV-1 Fusion Peptide Adopting an Extended Conformation: Dose and Sequence Effects

Francisca B. Pereira, Félix M. Goñi, Arturo Muga, and José L. Nieva
Biomembrane Laboratory/CSIC-Associated Unit, Department of Biochemistry and Molecular Biology, University of the Basque Country, 48080 Bilbao, Spain

ABSTRACT The peptide HIV_{arg}, corresponding to a sequence of 23 amino acid residues at the N-terminus of HIV-1 gp41 (LAV_{1a} strain), has the capacity to destabilize negatively charged large unilamellar vesicles. As revealed by infrared spectroscopy, the peptide associated with those vesicles showed conformational polymorphism: in the absence of cations the main structure was a pore-forming α-helix, whereas in the presence of Ca^{2+} the conformation switched to a fusogenic, predominantly extended β-type structure. Here we show that an extended structure can also be involved in electrically neutral vesicle destabilization induced by the HIV-1 fusion peptide when it binds the vesicle from the aqueous phase. In the absence of cations, neutral liposomes composed of phosphatidylcholine, phosphatidylethanolamine, and cholesterol (molar ratio 1:1:1) selected for an extended structure that became fusogenic in a dose-dependent fashion. At subfusogenic doses this structure caused the release of trapped 8-aminonaphtalene-1,3,6-trisulfonic acid sodium salt/*p*-xylenebis(pyridinium)bromide from liposomes, indicating the existence of a peptide-mediated membrane destabilizing process before and independent of the development of fusion. When compared to HIV_{arg}, the fusion activity of HIV_{ala} (bearing the R22 → A substitution) was reduced by 70%. Fusogenicity was completely abolished when a second substitution (V2 → E) was included to generate $HIV_{ala\text{-}E2}$, a sequence representing the N-terminus of an inactive gp41. However, the three sequences associated with vesicles to the same extent, and the three adopted a similar extended structure in the membrane. Whereas 1-(4-trimethylaminophenyl)-6-phenyl-1,3,5-hexatriene emission anisotropy was unaffected by the three peptides, DPH emission anisotropy in membranes was increased only by the fusogenic sequences. Taken together, our observations strongly argue that it is not an α-helical but an extended structure adopted by the HIV-1 fusion peptide what actively destabilizes cholesterol-containing, electrically neutral membranes. Moreover, membrane destabilization is modulated by the amino acid sequence in the extended structure. The effect displayed by the aforementioned V2 → E substitution suggests that the fusion process described here could be reflecting a physiologically relevant phenomenon.

INTRODUCTION

Initial exposure to the aqueous medium and further insertion into target cell membranes of fusion peptides are thought to play a central role in the fusion mechanism of a wide range of enveloped viruses (White, 1990; Zimmerberg et al., 1993). It has been proposed that during the entry process of HIV-1, its envelope protein gp120/41 exposes, upon activation, the fusion peptide at the N-terminal of gp41 transmembrane subunit (Gallaher, 1987; Moore et al., 1993). Activation of gp120/41 occurs after interaction with the primary receptor CD4 and requires the presence of human cofactors that have just recently been identified as chemokine receptors (Feng et al., 1996; Dragic et al., 1996). Extrusion of the peptide into the aqueous environment might also involve functional parts of gp41 that show the ability to oligomerize through the formation of coiled coils (Wild et al., 1994; Lu et al., 1995). On the basis of its hydrophobic character, it is also postulated that the interaction of the fusion peptide with the lipidic components of the cell membrane could eventually trigger the actual merging of the viral and cell membranes via a currently unknown mechanism (White, 1992).

Based on findings that support the involvement of the N-terminus of gp41 in human immunodeficiency virus (HIV) fusion in vivo (Bosch et al., 1989; Freed et al., 1990, 1992), several studies have been addressed to investigate the putative membrane-destabilizing effects of this sequence by using representative synthetic peptides and model membranes (Rafalski et al., 1990; Slepushkin et al., 1992; Gordon et al., 1992; Martin et al., 1993). We have reported that the HIV-1 fusion peptide behaves as a fusogen in vesicles consisting of negatively charged 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG) only when Ca^{2+} or Mg^{2+} is present in the medium (Nieva et al., 1994; Pereira et al., 1995). In the absence of cations, the peptide causes lysis of dispersed POPG LUVs, probably through the formation of discrete pores in the membrane. Most interestingly, we found that the peptide associated with POPG vesicles adopts different secondary structures, a phenomenon that appears to be likewise modulated by Ca^{2+}; i.e., in the presence of Ca^{2+}, under conditions supporting fusion, the peptide adopts mostly a β-structure, whereas in the absence of

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Address reprint requests to Dr. José Luis Nieva, Biomembrane Laboratory/CSIC-Associated Unit, Department of Biochemistry and Molecular Biology, University of the Basque Country, P.O. Box 644, 48080 Bilbao, Spain. Tel.: +34-4-4647700, ext. 2378; Fax: +34-4-4648500; E-mail: gbpniesj@lgehu.es.

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cations the pore-forming structure is represented by a predominant α-helical conformation.

The polymorphic conformational behavior detected for the HIV-1 fusion peptide in POPG membranes is not unique. The conformation of most membrane-interacting proteins is modulated by the host lipid bilayer (Surewicz et al., 1992; Bañuelos and Muga, 1995). In addition, a wide range of them appear to be conformationally flexible, and subtle differences in both the aqueous medium and the lipid environment can lead to quite different membrane-bound structures. Among others, examples of membrane-bound peptides and proteins for which conformational polymorphism has been detected are amphiphilic and hydrophobic signal peptides (Tamm, 1991), apolipoproteins (Taylor et al., 1993), synthetic model peptides (Lee et al., 1993), α-lactalbumin (Bañuelos and Muga, 1996), and α-hemolysin (Bakás et al., 1997).

The membrane environment into which the HIV-1 fusion peptide should partition in vivo is likely to be better represented by lipids devoid of a net charge (Aloia et al., 1993). Furthermore, cholesterol is known to be an important membrane component of both the virion and target cell membranes (Aloia et al., 1988, 1993). The HIV-1 envelope shows a high cholesterol-to-phospholipid molar ratio that confers on this membrane the low fluidity environment necessary for viral stability and infectivity. Consequently, in this work we have employed LUVs made of zwitterionic phospholipids and cholesterol as targets for the HIV-1 fusion peptide. Because the peptide could sample different conformations, depending on the lipid enviroment, we have evaluated its membrane-associated structure in this system by means of infrared spectroscopy. We conclude that the peptide switches from the α-helical conformation adopted in negatively charged membranes to an extended structure in neutral membranes containing cholesterol. Moreover, when folded into this extended structure, the peptide destabilizes isolated vesicles and induces fusion in a sequence-regulated manner, indicating that this conformation actively modifies the membrane architecture and that such ability may be modulated by its chemical structure.

MATERIALS AND METHODS

Materials

Dioleoylphosphatidylcholine, dioleoylphosphatidylethanolamine, and the fluorescent probes *N*-(7-nitro-benz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (N-NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (N-Rh-PE) were purchased from Avanti Polar Lipids (Birmingham, AL). 8-aminonaphtalene-1,3,6-trisulfonic acid sodium salt (ANTS), *p*-xylenebis(pyridinium)bromide (DPX), 1,6-diphenyl-1,3,5-hexatriene (DPH), and 1-(4-trimethylaminophenyl)-6-phenyl-1,3,5-hexatriene (TMADPH) were from Molecular Probes (Junction City, OR). Cholesterol (CHOL), biscinchoninic acid (BCA), and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade. The sequences representing the N-terminus of the HIV gp41, HIV_{arg} (AVGIGALFLGFLGAAGSTMGARS; LAV_{1a} strain), HIV_{ala} (AVGIGALFLGFLGAAGSTMGAAS; HTLV-III, BH10 clone), and $HIV_{ala\text{-}E2}$ (AEGIGALFLGFLGAAGSTMGAAS; HTLV-III, BH10 clone-41.2 mutant as defined by Freed et al., 1990) were synthesized as their C-terminal carboxamides and purified (estimated homogeneity >90%) by Quality Controlled Biochemicals (Hopkinton, MA). Peptide stock solutions were prepared in dimethylsulfoxide (DMSO) (spectroscopy grade).

Vesicle preparation and size determination

Large unilamellar vesicles (LUVs) consisting of dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE), and cholesterol (CHOL) (molar ratio, 1:1:1) were prepared according to the extrusion method of Hope et al. (1985) in 5 mM HEPES, 100 mM NaCl (pH 7.4) buffer. Internal and external osmolarities were adjusted by adding NaCl. The osmolarities of all solutions were measured in a cryoscopic osmometer (Osmomat 030; Gonotec, Berlin, Germany). Lipid concentrations of liposome suspensions were determined by phosphate analysis (Böttcher et al., 1961). Mean diameter and size distribution of vesicles before and after peptide addition were measured by quasielastic light scattering with a Malvern Zeta-Sizer instrument.

Fluorimetric assays for vesicle fusion and leakage

All fluorescence measurements were conducted at 37°C in thermostatically controlled cuvettes with a Perkin-Elmer LS50-B spectrofluorimeter. The medium in the cuvettes was continuously stirred to allow the rapid mixing of peptide and vesicles. Membrane lipid mixing was monitored using the resonance energy transfer (RET) assay, described by Struck et al. (1981). The assay is based on the dilution of N-NBD-PE and N-Rh-PE. Dilution due to membrane mixing results in an increase in N-NBD-PE fluorescence. Vesicles containing 0.6 mol% of each probe were mixed with unlabeled vesicles at a 1:4 ratio. 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 of the lipids in the system. The latter value was set by the fluorescence intensity of vesicles, labeled with 0.12 mol% of each of the fluorophores, at the same total lipid concentration as in the fusion assay. Release of vesicular contents to the medium was monitored by the ANTS/DPX assay. LUVs containing 12.5 mM ANTS, 45 mM DPX, 20 mM NaCl, and 5 mM HEPES (Ellens et al., 1985) 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 mOsm as described above. Fluorescence measurements were performed by setting the ANTS emission at 520 nm and the 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). Values for the final extent of leakage and fusion were obtained at times at which the kinetics of the processes leveled off. The shortest times considered were 5 min for peptide-to-lipid ratios higher than 1:100, and 10 min for lower ratios.

Peptide binding to vesicles

Peptide binding to vesicles was estimated by flotation analysis of the peptide-liposome complexes in D_2O buffer (5 mM HEPES, 100 mM NaCl, pH 7.4), as detailed by Pereira et al. (1995). Peptide dissolved in DMSO was added to 1 ml DOPC/DOPE/CHOL LUVs (1 mM) prepared in D_2O buffer (peptide-to-lipid ratio 1:100). After overnight incubation at room temperature, centrifugation of the peptide-lipid complexes in a Beckman Optima TLX ultracentrifuge in a TL100 rotor (627,000 $\times$ *g*, 120 min) gave rise to a homogeneous band of vesicles floating on top of the D_2O buffer. The tubes were fractionated from the top, and peptide distribution was

subsequently quantitated by the BCA assay, according to the instructions of the manufacturer. The peptide fraction cofloating with vesicles was considered to be membrane-bound.

Electron microscopy

Peptide-liposome complexes were subjected to flotation in D_2O buffer as described. Samples, collected from either the top (floating fraction) or bottom (pellet) of centrifuge tubes, were subsequently incubated in copper grids for 2 min, dried, and finally stained for 1 min with 1% uranyl acetate. Electron micrographs were obtained with a JEOL 1200EX II microscope operated at 120 kV and recorded on Kodak SO-163 film.

Infrared spectroscopy

Measurements were essentially conducted as done by Nieva et al. (1994). Samples consisted of peptide-lipid complexes obtained in D_2O buffer according to the above flotation protocol. Infrared spectra were recorded in a Nicolet 520 spectrometer equipped with a DTGS detector. Samples, containing ~4 mg peptide/ml, were placed between two CaF_2 windows separated by 50-μm spacers. Two hundred scans as a sample and 200 scans as a reference were taken for each spectrum, with a shuttle device. Spectra were transferred to a personal computer, with which solvent subtraction and band-position determinations were performed as previously reported (Arrondo et al., 1994).

Fluorescence anisotropy

Steady-state fluorescence anisotropy of DPH and TMADPH was measured in a MPF-66 Perkin-Elmer fluorometer. Excitation was at 360 nm and emission was recorded at 430 nm. A 390-nm cutoff filter was used to prevent scattered light from reaching the detector. The expression used to calculate anisotropy (R) contained the grating correction factor (Lakowicz, 1983). Sample turbidity caused a negligible effect on the anisotropy values.

RESULTS

HIV_{arg}-induced destabilization of DOPC/DOPE/CHOL vesicles

We first carried out a simultaneous characterization of the peptide-induced membrane destabilization process (leakage, fusion, and size increase) and adopted secondary structure (infrared spectroscopy). HIV_{arg}-induced leakage and fusion were detected by the dilution of coencapsulated fluorescent probes into the medium and the mixing of membrane lipids, respectively. Leakage of vesicles induced by peptide addition reflects peptide-membrane interaction. In Fig. 1 it is shown that HIV_{arg} readily induced the leakage of small molecules. Leakage was monitored by dequenching of ANTS due to its release from ANTS/DPX containing vesicles. In the same figure, HIV_{arg} induced-membrane mixing is demonstrated by employing the RET assay (Struck et al., 1981), which monitors the increase in NBD fluorescence after probe dilution from labeled to unlabeled vesicles in a 1:4 population. Similar results (data not shown) were obtained by using an assay based on excimer formation by the lipidic probe Pyrene-PC (Pal et al., 1988). Thus a fluorophore located at the membrane interface and a fluorophore immersed in the hydrocarbon matrix monitored essentially the same lipid mixing event. It is important to note that this process was induced by HIV_{arg} in the absence of cations as opposed to the case of POPG vesicles that only fused when Ca^{2+} or Mg^{2+} was present in the medium (Nieva et al., 1994).

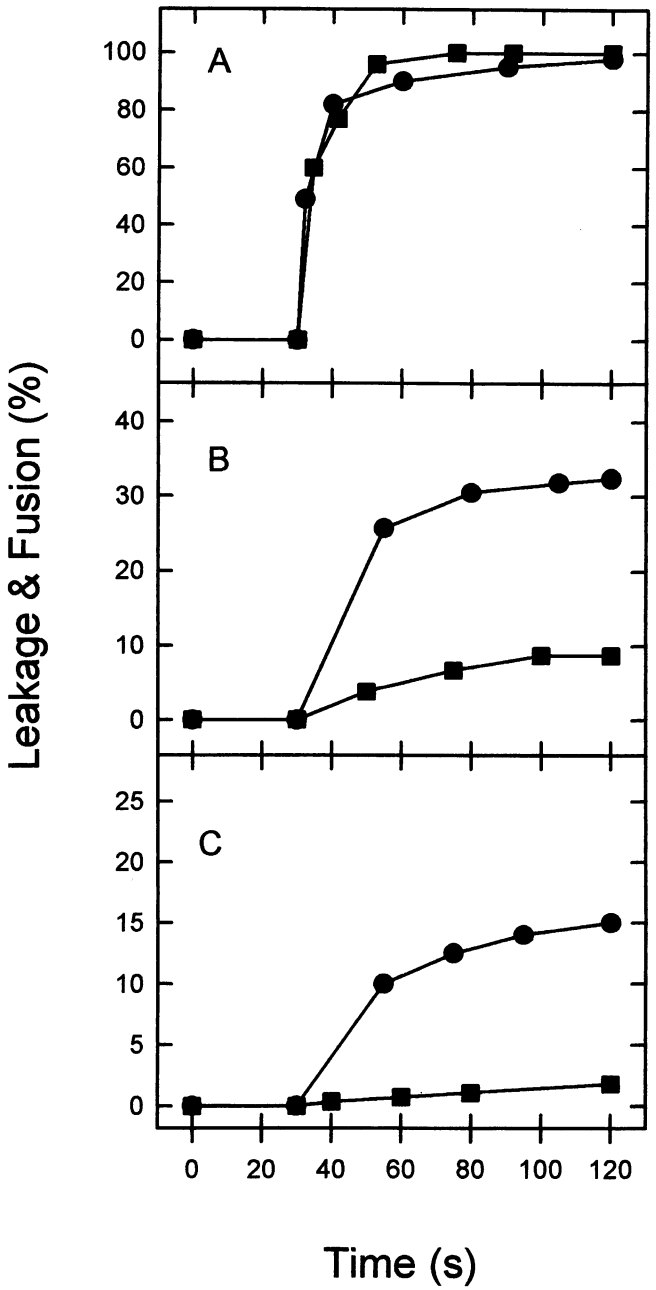


FIGURE 1 HIV_{arg} fusion peptide-induced DOPC/DOPE/CHOL LUV destabilization. The peptide was added to vesicle suspensions at 37°C, and its effects on vesicle stability were compared. Vesicle concentration was 100 μM. Peptide was added at peptide-to-lipid ratios of 1:10 (A), 1:100 (B), and 1:275 (C). ●, Kinetics of leakage of ANTS/DPX. ■, Kinetics of membrane lipid mixing (resonance energy transfer assay).

From these data we can conclude that in the absence of cations, HIV_{arg} interacted with DOPC/DOPE/CHOL membranes and induced two types of destabilizations, one leading to the rupture of the permeability barrier and the other to

fusion. From kinetic traces such as the ones displayed in Fig. 1, we consistently obtained higher initial leakage rates than lipid mixing rates at a fixed peptide-to-lipid ratio. In principle, this could either indicate that permeabilization preceded fusion in our system or merely reflect differences inherent in the fluorimetric assays. However, the peptide-dose effect confirmed that leakage was an event that could evolve independently of fusion and probably precede it (Fig. 1 *C*). The dose effect is better illustrated when we compare the final extents of both processes as a function of the peptide-to-lipid ratio. Fig. 2 *A* shows the increase in the final extents (see Materials and Methods) of membrane mixing as compared to leakage of contents with increasing amounts of peptide. We found that a 1:35 peptide-to-lipid ratio was required to induce 20% lipid mixing. In comparison, HIV_{arg} induced 20% leakage at a much lower 1:275 peptide-to-lipid ratio. In other words, fusion as compared to leakage probably had to meet additional requirements in terms of the amount of bound peptide per vesicle to evolve. Quasielastic light scattering results further demonstrated the existence of peptide-induced leakage in the absence of vesicle size increase. As shown in Fig. 2 *A*, the increase in vesicle size paralleled the increase in the amount of fusion. Increase in particle mean size in a vesicular system can arise from simple aggregation and/or fusion. From the data in Fig. 2 *A* we can deduce that the peptide was able to interact with isolated vesicles and induce leakage of their contents. Therefore, permeabilization did not appear to be promoted as a consequence of mechanical strains imposed by vesicle aggregation or a leaky fusion process induced by the peptide.

With the aim of elucidating the correct correlation between the structure adopted by the peptide in the membrane and the occurrence of leakage, fusion, and size increase, structural studies were performed on peptide-liposome complexes prepared by mimicking as much as possible the assay conditions described in Fig. 2 *A*. Thus HIV_{arg} was added at 37°C to DOPC/DOPE/CHOL LUV suspensions with continuous stirring. After incubation, the mixtures prepared in D_2O buffer were subjected to centrifugation to separate free peptide from floating vesicles containing the bound peptide (see below). Fig. 2 *B* shows the conformation-sensitive amide I region of the infrared spectra of peptides incorporated into DOPC/DOPE/CHOL membranes at the approximate doses that activate leakage (*curve a*), fusion (*curve b*), and both processes simultaneously (*curve c*). The spectra display an intense absorption maximum at 1625 cm^{-1} and a less intense maximum at 1689 cm^{-1}. The ability of Fourier transform infrared (FTIR) spectroscopy to detect and discriminate β-like conformations from others (Arrondo et al., 1993; Surewicz et al., 1993) unambiguously indicates that the peptide occurs in an extended conformation. Whether it is a classical antiparallel β-sheet, or we are dealing with intermolecularly H-bonded aggregates of extended structure, cannot be said with certainty. The fact that similar spectra are found for initial peptide-to-lipid ratios (before flotation) of 1:800, 1:200, and 1:50 demonstrates that the composition of the system does not influence peptide conformation. Thus the peptide, in these samples, adopted the same predominant extended structure under conditions where only leakage or, alternatively, both leakage and fusion can occur (see Fig. 2). The spectra also display an intense band centered at 1734 cm^{-1} that corresponds to the C=O stretching vibration of the phospholipid ester bonds.

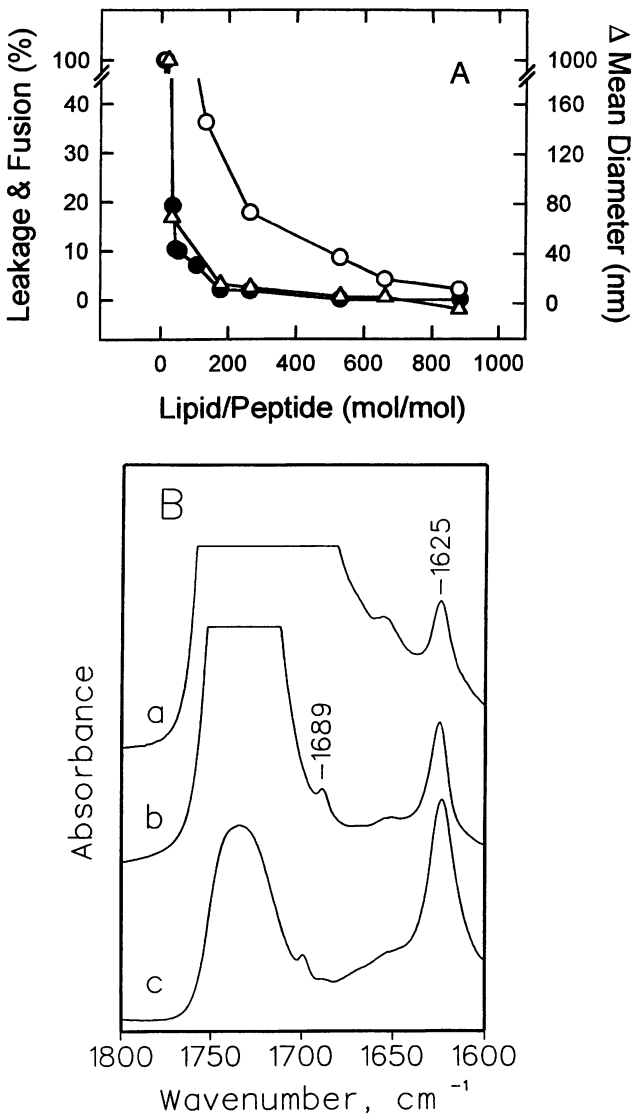


FIGURE 2 (*A*) Final extents of leakage and fusion (see Materials and Methods) and concomitant increase of vesicle mean size as a function of increasing concentrations of HIV_{arg}. ●, Membrane mixing; ○, leakage: △, increase in size. Vesicle concentration was 100 μM. (*B*) Fourier transform infrared (FTIR) spectra of HIV_{arg} incorporated into DOPC/DOPE/CHOL membranes after external partition from the aqueous phase. The peptide was added to LUVs at different peptide-to-lipid ratios under the experimental conditions used for the fusion and leakage assays (see Materials and Methods). Peptide-lipid complexes were subsequently isolated from the floating fractions after ultracentrifugation in D_2O buffer. *Curve a*: 1:800 peptide-to-lipid ratio; *curve b*: 1:200 peptide-to-lipid ratio; *curve c*: 1:50 peptide-to-lipid ratio. The spectra are not drawn to the same scale, to better show the amide I region (1700–1600 cm^{-1})

To further ascertain whether the structural data were recorded on the peptide population associated with the

vesicles, we characterized, by means of electron microscopy, the resulting fractions after flotation of the peptide-lipid samples (Fig. 3). As shown by the electron micrographs, the unbound peptide in the precipitates consisted of fibrillar bundles ~500 nm in diameter (Fig. 3 *A*). The formation of these aggregates could be at the origin of peptide inactivation in solution. The results in Fig. 4 illustrate the effect of incubating the peptide in solution before the addition of vesicles on the extent of fusion induced by HIV_{arg}. After a 30-s incubation in solution, the extent of lipid mixing induced by the peptide was reduced to less than one-third, and after 2 min fusion became residual. We conclude that unbound HIV_{arg} gave rise to aggregates constituting an inactive peptide population. Micrographs of floating fractions are also shown in Fig. 3, *B* and *C*. The control in the absence of HIV_{arg} shows vesicles of a size in the range of 100 nm (Fig. 3 *B*). In contrast, the floating fraction of samples containing the peptide reveals the presence of larger vesicles (Fig. 3 *C*). Importantly, in the latter sample, no peptide aggregates were seen to be floating with vesicles. Hence the peptide associated with these vesicles must adopt discrete structures in membranes that are not discernible at the experimental magnification. Peptides in these samples were considered to represent the membrane-bound fraction and were consequently subjected to structural characterization as described above.

Sequence substitutions: effects on fusion, conformation, and membrane interaction

The effect of single substitutions within the peptide sequence on fusion and secondary structure of the membrane-bound peptide was investigated next. R22xA substitution was located in a position close to the C-terminus of the peptide and yielded HIV_{ala}, which represents the fusion peptide of the BH10 clone derived from the HTLV-III strain. In principle, this peptide should be fusogenic. A further V2xE substitution in the HIV_{ala} N-terminus yields $HIV_{ala\text{-}E2}$, a fusion peptide representing the 41.2 inactive mutant described by Freed et al. (1990, 1992). This substitution should cause inactivation of the peptide if our assay were reflecting any physiologically relevant interaction.

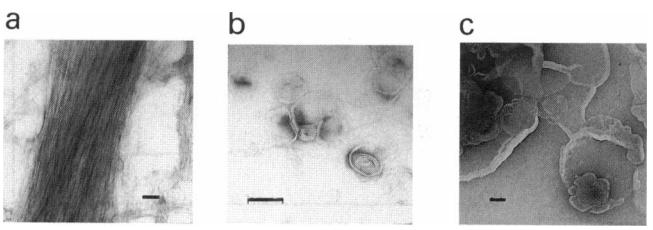


FIGURE 3 Electron micrographs of the products obtained after flotation of peptide-vesicle mixtures in D_2O buffer. Vesicles (1 mM) were mixed with HIV_{arg} at a peptide-to-lipid ratio of 1:10, incubated overnight, and subjected to ultracentrifugation (2 h at 600,000 × *g*). (*a*) Pellet fraction; (*b*) control, showing floating vesicles in the absence of peptide addition; (*c*) floating vesicles after incubation with the peptide. Bar = 100 nm.

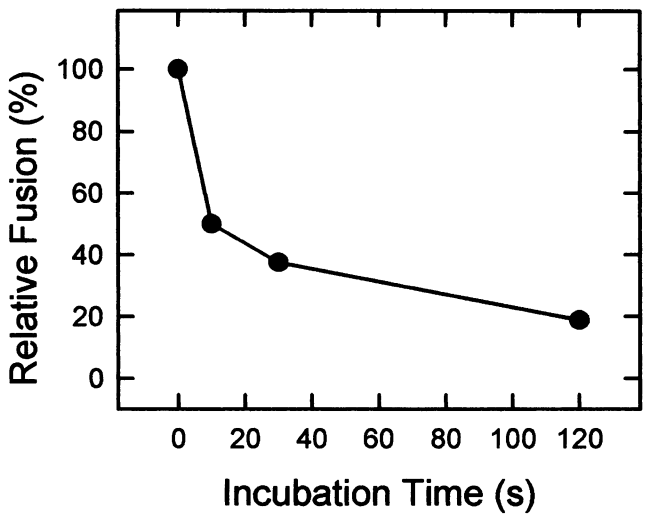
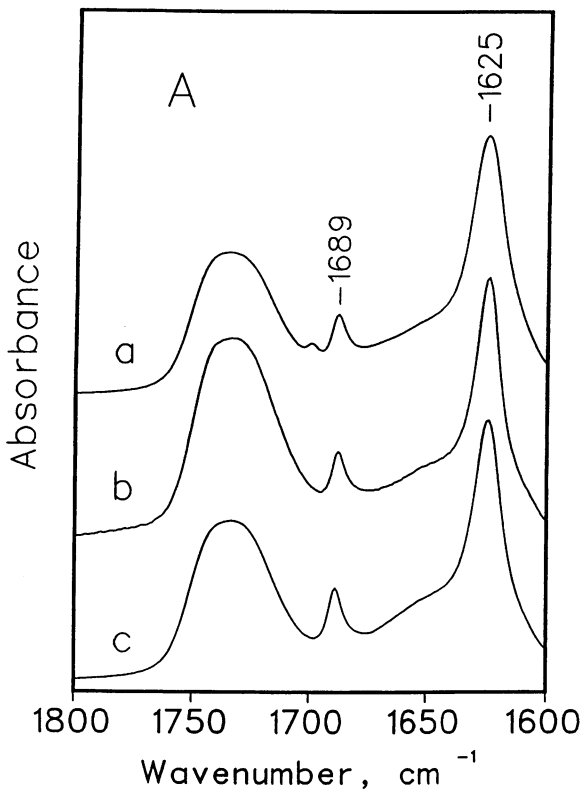


FIGURE 4 HIV_{arg} inactivation in solution. The peptide (2 μM) was incubated at increasing time periods in buffer before vesicle addition (100 μM), and the final extent of the remaining lipid mixing activity was monitored.

Fig. 5 shows the fusion assays in the presence of DOPC/DOPE/CHOL LUVs and each of these three peptides at a 1:10 peptide-to-lipid molar ratio, as well as their corresponding membrane-bound secondary structure. FTIR results in Fig. 5 *A* demonstrate that in vesicles the three peptides adopted the same predominant extended structure. Moreover, from these spectra it can be estimated that the three peptides bound to vesicles to a similar extent, because the relative areas of the 1734 cm^{-1} (phospholipids) and amide I (peptide) bands are roughly the same for the three samples. The equivalent binding capacity of the three peptides was confirmed by the BCA assay described in Materials and Methods. According to this assay, at a peptide-to-lipid ratio of 1:100, 82% of HIV_{arg}, 72% of HIV_{ala}, and 72.5% of $HIV_{ala\text{-}E2}$ bound to vesicles. Lipid mixing kinetics are compared in Fig. 5 *B*. HIV_{arg} induced extensive and fast fusion (initial rate 10%/s; final extent 100%). In comparison, HIV_{ala} displayed a reduced fusogenic activity (initial rate 0.2%/s; final extent 35%), and $HIV_{ala\text{-}E2}$ did not induce fusion at all, judging from the absence of NBD dequenching for up to 10 min. In conclusion, peptides that bound lipid vesicles to the same extent, adopting essentially identical secondary structures, displayed completely different fusion activities.

Thus the above results demonstrate that even if a predominant extended structure represented the fusion-competent version in neutral membranes, the ability to adopt this secondary structure by HIV-1 fusion peptides did not correlate with their fusion capacity. Other factors must be invoked to explain their dissimilar fusogenicities. In an attempt to find an explanation for the differences, we looked next at the effects of the peptides on the structure of the lipid bilayer.

Information from the interface and hydrocarbon region of the lipid bilayer was obtained by measuring, respectively,



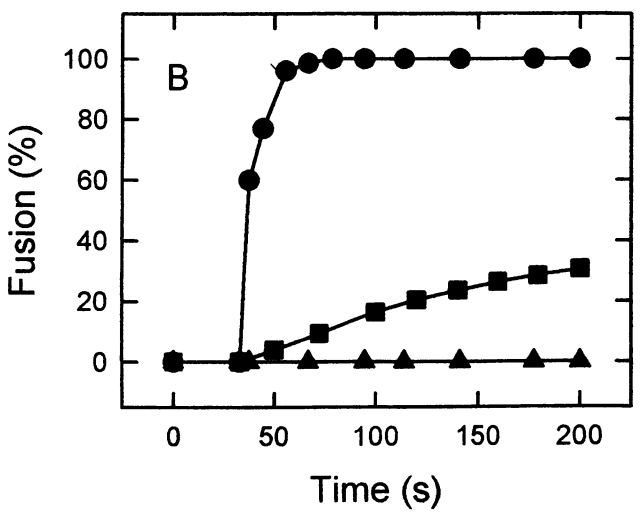


FIGURE 5 Effect of sequence substitutions on fusion activity and membrane-associated secondary structure. (*A*) FTIR spectra of the vesicle-associated peptides, HIV_{arg} (*a*), HIV_{ala} (*b*), and $HIV_{ala\text{-}E2}$ (*c*). The peptides were added to vesicles at a peptide-to-lipid ratio of 1:10. (*B*) Time courses of membrane mixing induced by HIV_{arg} (●), HIV_{ala} (■), and $HIV_{ala\text{-}E2}$ (▲) in DOPC/DOPE/CHOL vesicles.

the anisotropy of the fluorescence emission of DPH and that its cationic analog TMADPH (Haugland, 1996) as a function of increasing amounts of peptide. The trimethylammonium substituent in TMADPH acts as a surface anchor that locates the probe close to the membrane interface. DPH, on the other hand, resides primarily in the membrane interior, sensing the environment of the phospholipid acyl chains. Results in Fig. 6 *A* demonstrate that TMADPH emission anisotropy was almost unaffected by the presence of any of the peptides. By contrast, as shown in Fig. 6 *B*, DPH anisotropy was increased by the presence of HIV_{arg} and HIV_{ala}, but not by the presence of $HIV_{ala\text{-}E2}$. Therefore, emission anisotropy by the probe located within the hydrocarbon matrix was affected exclusively by the fusogenic peptides. Increase in anisotropy of DPH fluorescence can be explained in terms of restriction to the extent and/or rate of the rotational diffusion (Lakowicz, 1983). The movement restriction in the DPH environment may indicate that only the fusogenic peptides penetrated into the membrane to the level of the hydrocarbon chains (Jones and Gierasch, 1994).

DISCUSSION

Membrane destabilization and adopted conformation

It seems likely that many conformational changes taking place in the HIV-1 spike protein gp120/41 during the entry process are mainly needed to ensure the extrusion into the aqueous phase of the fusion peptide in the vicinity of the target membrane (Moore et al., 1993). Once exposed, the

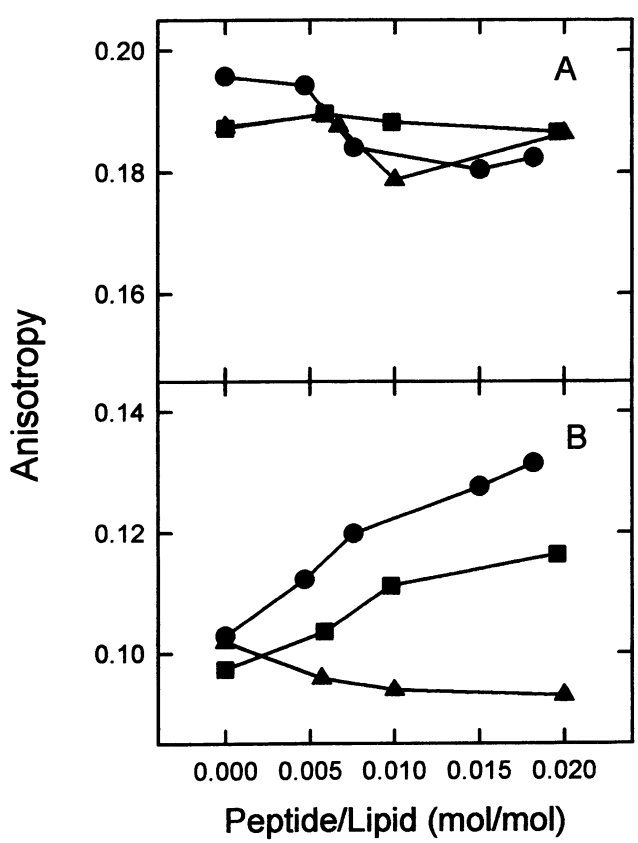


FIGURE 6 TMADPH (*A*) and DPH (*B*) emission anisotropy as a function of the peptide-to-lipid ratio. Peptides were added to vesicles (100 μM) containing the probes. HIV_{arg} (●), HIV_{ala} (■), and $HIV_{ala\text{-}E2}$ (▲).

peptide probably must partition into the lipidic core of the cell membrane to carry out its putative destabilizing function. One important aspect that has been considered in the present study is the necessity of mimicking the in vivo situation as much as possible to characterize the HIV_{arg}-membrane interaction. Accordingly, we have paid attention to several issues. First, the peptide must partition from the aqueous phase into the membrane to account for the possible effects of the bilayer interface on the adopted conformation and to exert an eventual membrane destabilization (Deber and Goto, 1996). Hence we looked at the peptide-membrane interaction after the external addition of the peptide to vesicle suspensions. Second, we used LUVs as targets because the curvature of these vesicles closely resembles that of the plasma membrane. Third, the vesicles were made of a mixture of phospholipids and cholesterol representing the uncharged fraction of the putative target composition. Finally, in an effort to better correlate structure and function, structural studies were performed on peptides interacting with membranes under functional conditions, i.e., those at which peptide-induced leakage and fusion occur. To ensure that we precisely studied the structure adopted by the membrane-bound peptide population, we followed a strategy to separate by flotation the unbound peptide aggregates that most likely represent an inactive fraction (Fig. 3). Nevertheless, it should be pointed out that the model system described here is still far from entirely reflecting the in vivo complexity of the studied interaction. Factors like additional functionally relevant domains in gp41, membrane asymmetry, or transmembrane electrochemical gradients could also be affecting the interaction of the HIV-1 fusion peptide with its target membrane under physiological conditions.

From the results in Figs. 1 and 2 it can be deduced that HIV_{arg} interaction with neutral vesicles made of DOPC/DOPE/CHOL induces permeabilization (leakage) and fusion (lipid mixing). In contrast to negatively charged POPG vesicles (Nieva et al., 1994; Pereira et al., 1995), fusion did not require the presence of cations to occur in suspensions of DOPC/DOPE/CHOL vesicles. In POPG vesicles, peptide-induced leakage developed in the absence of cations as well. However, the leakage detected in the DOPC/DOPE/CHOL LUV system in the absence of cations seems to represent a different process. The leakage process in DOPC/DOPE/CHOL LUV is induced by peptides adopting an extended conformation in the membrane (Fig. 5), whereas in POPG vesicles the adopted structure is predominantly α-helix (Nieva et al., 1994; Pereira et al., 1995). We thus speculate that the initial leakage in the absence of aggregation and fusion that occurs in DOPC/DOPE/CHOL LUV (Fig. 2) might be the expression of the initial membrane destabilization that leads to fusion being part of the same process. Bilayer destabilization probably gains intensity upon binding of more peptides per vesicle, until vesicle aggregation and eventual fusion ensue. In contrast to the possible consideration of the peptide as a mere aggregating device that puts into contact inherently unstable bilayers, the aforementioned leakage process occurring in isolated vesicles suggests that the peptide is the agent conferring instability on the membrane, so that fusion can eventually take place. The observations of DPH polarization (Fig. 6) would also support this point of view. Moreover, recent observations by our group argue that the peptide is able to alter the phase behavior of the lipids, probably by modifying the membrane intrinsic curvature (Pereira et al., manuscript in preparation).

Under equilibrium conditions, HIV_{arg} bound to neutral DOPC/DOPE/CHOL vesicles displays a FTIR spectrum compatible with the majority of the peptide being arranged as an extended structure (Fig. 2 *B*). Under equivalent measuring conditions (i.e., in the absence of cations), HIV_{arg} bound to negatively charged POPG vesicles adopted a predominantly α-helix conformation (Nieva et al., 1994; Pereira et al., 1995). Thus the peptide shows conformational polymorphism in the sense that, depending on vesicle composition, the peptide may take on different secondary structures. Selection of distinct conformations depending on the lipidic environment is characteristic of many membrane-interacting peptides and proteins (Surewicz et al., 1992). This effect is probably modulated in our system by the vesicle surface charge rather than by the presence of defined lipids, because neutralization of POPG vesicles by Ca^{2+} addition induces the adoption of a β-structure by the peptide (Nieva et al., 1994; Pereira et al., 1995). It is well known that surface pH in negatively charged vesicles is lower than in bulk solution and that this lower pH can modulate as well the conformation of bound proteins. Therefore we also studied the conformation adopted by peptides bound to uncharged vesicles at pH 5.0. The amide I bands in the collected spectra at pH 5.0 and pH 7.4 were almost identical (data not shown), indicating that a pH-driven transition from an extended to an α-helical conformation is not likely in our system.

Fig. 2 *B* also illustrates that the main secondary structure did not change with the amount of peptide bound to DOPC/DOPE/CHOL vesicles. This result apparently contradicts previous FTIR determinations by Rafalsky et al. (1990) and Gordon et al. (1992) indicating that the peptide is able to adopt an α-helical arrangement in related membranes at low doses. Even though subtle differences in membrane composition could explain this discrepancy (Rafalsky et al. used pure POPC vesicles and Gordon et al. isolated lipids, including the acidic fraction, from red blood cell ghosts), we think that the relevant factor might be the sample preparation method (the above authors' cosolubilized peptide and lipid before solvent evaporation and hydration, instead of the method applied in our case).

We conclude that the above-described predominantly extended structure represents the fusion-active conformation of HIV_{arg} in DOPC/DOPE/CHOL vesicles. It should be noted that the FTIR spectra are recorded after fusion has been completed, and therefore we cannot rule out the existence of transient conformations, other than the detected extended structure, that might be involved in the fusion

process. The prevailing assumption that the active conformation of viral fusion peptides was in all cases an α-helical arrangement (Lear and DeGrado, 1987; Harter et al., 1989; Takahashi, 1990; Rafalski et al., 1990) was initially questioned by Gallaher et al. (1992). Since then, additional evidence suggesting a more complex scenario has become available. Epand et al. (1992) deduced that the measles virus fusion peptide adopts mostly a β-structure in a lipid environment. Our results on HIV_{arg} interacting with POPG LUV in the presence of Ca^{2+} indicated that a main β-structure represents the equilibrium conformation after fusion has been completed (Nieva et al., 1994; Pereira et al., 1995). Muga et al. (1994) reported that the putative fusion peptide of PH-30, a sperm surface protein involved in sperm-egg fusion, also adopts a predominant β-conformation when associated with membranes. Interestingly, these sequences were initially modeled as sided membrane-interactive α-helices (White, 1992). More recently, Gray et al. (1996) have shown by means of combining FTIR and circular dichroism data that synthetic peptides corresponding to the wild-type or to several fusogenic and nonfusogenic mutants of influenza fusion peptide (Steinhauer et al., 1995) contained segments of α-helical and β-strand conformation. This relatively new notion of a short amino acid sequence that adopts a β-type conformation being able to destabilize membranes is further supported by recent data obtained for the antimicrobial peptides defensins interacting with vesicles (White et al., 1995).

In our case FTIR spectroscopy suggests that most probably the peptide monomers arrange into extended structures that may be stabilized by either inter- or intramolecular hydrogen bonding. Formation of the latter structures could be promoted by interfacial binding of peptide monomers. In fact, when, to detect sequence segments that could show a tendency to partition into membrane interfaces, we applied the hydrophobicity scale developed by Wimley and White (1996) to our peptide, we found a maximum located between L7 and L12 (Fig. 7). This internal sequence, containing two phenylalanines, could be involved in driving the peptide partition into the membrane interface.

Membrane destabilization and sequence effects

Correct configuration of the fusion peptide N-terminus appears to be crucial for the fusogenic function of certain spike proteins (Freed et al., 1992; Steinhauer et al., 1995). Several authors have located the N-terminus immersed in the membrane core after peptide-bilayer interaction (Gordon et al., 1992; Lüneberg et al., 1995). Our results also point out the importance of the N-terminus for the HIV-1 fusion peptide interaction with DOPC/DOPE/CHOL LUVs. The polar amino acid substitution V → E in position 2, known to block gp41 activity in vivo (Freed et al., 1992), renders the peptide unable to fuse neutral membranes. We previously reported a similar effect in POPG vesicles (Pereira et al., 1995). The absence of $HIV_{ala\text{-}E2}$-induced POPG fusion was correlated with its inability to adopt in the membrane the β-structure adopted by HIV_{ala} under conditions allowing this process (i.e., in the presence of 5 mM Ca^{2+}). By contrast, in the present work, nonfusogenic $HIV_{ala\text{-}E2}$ peptide in DOPC/DOPE/CHOL LUVs adopted exactly the same secondary structure as fusogenic HIV_{arg} and HIV_{ala} (Fig. 5). Thus the inability to adopt an extended β-strand secondary structure does not explain in this system the fusion inability displayed by $HIV_{ala\text{-}E2}$. There must be additional factors that explain fusogenicity by HIV_{arg} and HIV_{ala} in comparison with $HIV_{ala\text{-}E2}$.

Peptides arranged in an extended structure are likely to be surface-bound to bilayers. However, several findings indicate that the β-structure adopted by HIV_{arg} and HIV_{ala} probably penetrates to a certain degree into the bilayer matrix, whereas the one adopted by $HIV_{ala\text{-}E2}$ remains associated with the vesicle surface. Induction of leakage of aqueous contents from isolated vesicles (Fig. 2) suggests that the permeability barrier must be altered by a local

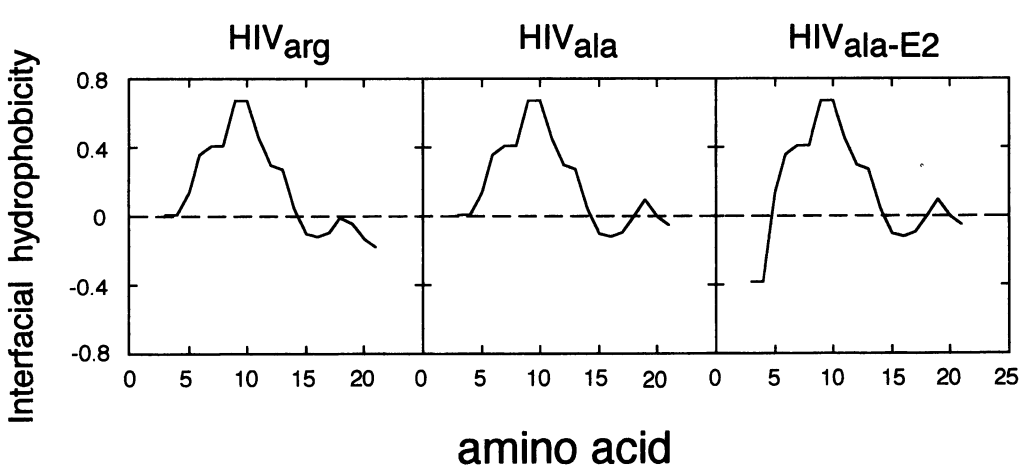


FIGURE 7 Hydropathy plots for HIV_{arg}, HIV_{ala}, and $HIV_{ala\text{-}E2}$. A window of five amino acids was used with the hydrophobicity scale at membrane interfaces determined by Wimley and White (1996).

disruption. It is difficult to envisage a molecular mechanism to describe such a disruption that would not imply a certain degree of peptide penetration. The ability to induce leakage probably correlates with fusogenicity, because nonfusogenic HIV_{ala-E2} was also unable to induce leakage of contents at any tested peptide-to-lipid ratio (data not shown).

Moreover, fluorescence anisotropy data indicate that HIV_{arg} and HIV_{ala} (but not HIV_{ala-E2}) increase emission anisotropy of the hydrophobic core-residing DPH probe. In a fluid phase, surface interaction of peptides might cause hindrance in DPH motion through a process of lateral lipid condensation, i.e., lipid-domain formation. However, such a process is unlikely to occur in our system, given the composition of both model membranes and peptides. In addition, such a superficial interaction would imply a large effect on the interface-anchored TMADPH emission anisotropy. As the results displayed in Fig. 6 indicate, that is not the case. Alternatively, acyl chain motion can be directly affected by inserted peptides. Signal peptides of the outer membrane protein A of *Escherichia coli* that have been shown to insert into membranes by fluorescence quenching of Trp (Hoyt and Gierasch, 1991), and to adopt a transmembrane orientation by spin-label electron spin resonance spectroscopy (Sankaram and Jones, 1994), increase DPH anisotropy in fluid membranes (Hoyt and Gierasch, 1991; Jones and Gierasch, 1994). An increase in DPH emission anisotropy correlates with a transmembrane orientation adopted only by the peptides representing the functional signal sequences (Jones and Gierasch, 1994). Assuming that membrane-interacting short sequences may affect DPH anisotropy in a similar fashion, our observations in the case of HIV-1 fusion peptides interacting with DOPC/DOPE/CHOL vesicles would support the finding that fusion-inducing peptides adopting a predominant β-structure penetrate into the membrane to the level of the phospholipid acyl chains. The ability to penetrate into the hydrophobic core would emerge as an important factor that might correlate with fusogenicity in our system. This would also explain, at least in part, the profound effect of the chemical structure on the fusogenicity of a peptide adopting a given secondary structure.

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