pH-Induced destabilization of lipid bilayers by a lipopeptide derived from influenza hemagglutinin

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Abstract

A synthetic twenty-one amino acid peptide (AcE4K) based on the amino acid sequence of the influenza HA2 fusion peptide was coupled to a distearoylglycerol lipid anchor by amidation of an N-terminal lysine side chain. The secondary structure of Lipo-AcE4K incorporated into POPC (1-palmitoyl-2-oleoyl-*sn*-phosphatidylcholine) liposomes was not measurably affected by pH, but increased membrane penetration was indicated by tryptophan fluorescence. At outer monolayer concentrations up to 10 mol%, Lipo-AcE4K formed stable liposomes with POPC and EPC/Chol (egg phosphatidylcholine/cholesterol) (55:45) at pH 7.5. Acid-induced destabilization and fusion of these vesicles were demonstrated by fluorescent lipid mixing and contents leakage assays, and by freeze-fracture electron microscopy. Membrane destabilization increased with increasing lipopeptide concentrations, decreasing pH, inclusion of cholesterol, and incorporation of lipopeptide into the inner monolayer as well as the outer monolayer of the liposomes. Fusion of liposomes bearing Lipo-AcE4K with erythrocyte ghosts was demonstrated by lipid mixing and fluorescence microscopy.

Keywords: Membrane fusion; Liposome; Lipopeptide; Influenza; Fluorescence microscopy; Erythrocyte

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1. Introduction

Cellular infection by enveloped viruses is a multi-step process in which fusion between the viral envelope and either the plasma membrane or an endosomal membrane of the cell is an essential component [1,2]. The process is mediated by viral glycoproteins which are responsible for binding to receptors on target cells, bringing the membranes into close proximity, and promoting the fusion of the viral envelope with the cellular membrane, leading to release of the viral capsid into the cytosol. A common feature of the viral fusion proteins is the presence of a short hydrophobic peptide which has the potential to interact with the membrane of a target cell. It is believed that these 'fusion peptides' penetrate cellular membranes and play a role in membrane destabilization and fusion. The functional role of fusion peptides has been investigated by site-directed mutagenesis of fusion proteins [3,4] and by physical characterizations of synthetic peptides with corresponding amino acid sequences and of their interactions with lipid bilayers [5–7].

The membrane-destabilizing properties of synthetic peptides based on the N-terminal sequences of the HA2 subunit of influenza hemagglutinin (HA) from various strains of the virus have been given considerable attention [8–12]. Membrane destabilization has been measured by hemolysis or leakage of lipid vesicles, and membrane fusion has been demonstrated by lipid mixing assays. In general, the peptides adopt amphipathic α-helical structures and penetrate lipid bilayers at the pH corresponding to that required for fusion of the native virus with the endosomal membrane. For peptides derived from the X31 and A/PR/8/34 strains the helical structure only forms upon neutralization of the acidic residues. However, the rank in importance of the degree of α-helical structure formed by the peptide, its overall hydrophobicity, and the orientation it adopts with respect to the lipid bilayer remains contentious.

Artificial amphipathic α-helical peptides which mimic the behaviour of viral fusion peptides have been synthesized and anchored to lipid bilayers by either a N-terminal palmitoyl moiety [13] or a C-terminal cysteine linked to a bifunctional phosphatidylethanolamine derivative [14]. These lipopeptides provide a means to study to what extent membrane fusion between lipid vesicles can be induced by a short membrane-bound peptide.

To study the properties of a membrane-anchored peptide derived from the HA2 N-terminal fusion peptide, we have synthesized the peptide AcE4K. The amino acid sequence is based on the peptide E4 studied by Wilschut and co-workers [10], which corresponds to the HA2 N-terminal sequence of an X31 mutant produced by [3]. The more recent study showed that under mildly acidic conditions E4 caused somewhat lower rates of contents leakage from lipid vesicles compared to wt. However, E4 exhibited a number of potentially advantageous properties: higher solubility at neutral pH, a more highly α-helical structure at low pH, and a greater response to changes in pH in terms of its propensity to insert into a lipid bilayer. In synthesizing AcE4K we acetylated the N-terminus and added a lysine residue to the C-terminus to permit a facile coupling to a reactive distearoylglycerol lipid anchor via the lysine side chain.

wt:	GLFGAIAGFIENGWEGMIDG
E4:	GLF**E**AIAGFIENGWEGMIDG
AcE4K:	**Ac**-GLF**E**AIAGFIENGWEGMIDG**K**

The resulting lipopeptide, Lipo-AcE4K, was incorporated into stable POPC (1-palmitoyl-2-oleoyl-*sn*-phosphatidylcholine), and EPC/Chol (egg phosphatidylcholine/cholesterol 55:45) liposomes at up to 10 mol% at pH 7.5. Acid-induced changes in peptide structure and lipid bilayer interactions as well as the resulting membrane destabilization and fusion were investigated.

2. Materials and methods

2.1. Lipids and chemicals

Crude AcE4K peptide was initially obtained from the laboratory of Dr. Ian Clark-Lewis, Biomedical Research Laboratory, University of British Columbia. Subsequently, purified peptide was purchased from Multiple Peptide Systems (San Diego, CA). 1,2-Distearoyl-*sn*-glycerol (DSG) was obtained from Genzyme (Boston, MA). 1-Palmitoyl-2-oleoyl-*sn*-phosphatidylcholine (POPC, egg phosphatidylcholine (EPC), *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-1,2-dioleoyl-*sn*-phosphatidylethanolamine (NBD-PE), and *N*-(lissamine rhodamine B sulfonyl)-1,2-dioleoyl-*sn*-
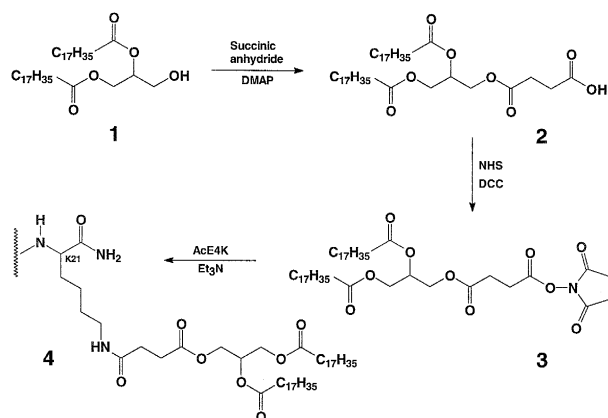
Fig. 1. Structure and synthesis of the lipopeptide Lipo-AcE4K (**4**). 1,2-*sn*-Distearoylglycerol (**1**) was elongated as a succinate ester (**2**), which was then converted to the activated succinimide (**3**). The activated lipid reacts exclusively with the primary amine on the C-terminal lysine of the peptide.

phosphatidylethanolamine (Rh-PE) were supplied by Avanti Polar Lipids (Alabaster, AL). Aminonaphthalenetrisulfonic acid (ANTS) and *p*-xylylene bis-(pyridinium) bromide (DPX) were purchased from Molecular Probes (Eugene, OR). Succinic anhydride, 4-dimethylaminopyridine, *N*-hydroxysuccinimide, dicyclohexylcarbodiimide, cholesterol and all buffers were purchased from Sigma (St. Louis, MO). All organic solvents were HPLC-grade and were used without redistillation.

2.2. Preparation of AcE4K and Lipo-AcE4K

After lyophilization, the peptide was purified by reverse-phase HPLC on a Synchropak (Synchrom) C8 semi-preparative HPLC column using a 40–70% linear gradient of acetonitrile in water (0.1% TFA) with a flow rate of 6 ml/min over 20 min. The peptide elutes at approximately 55% acetonitrile. The composition of the peptide was verified by amino acid analysis, mass spectrometry, and HPLC. Purity was estimated to be greater than 95%.

The synthesis of the lipopeptide is illustrated in Fig. 1. One gram of DSG (1.6 mmol) (**1**), 0.2 g succinic anhydride (2 mmol), and 0.24 g 4-dimethyl-aminopyridine (2 mmol) were dissolved in 10 ml of CH_2Cl_2 and stirred at room temperature for 1 h. The resulting acid (**2**) was isolated by removing solvent by rotary evaporation followed by purification by silica gel chromatography using 10% ethyl acetate in hexane as eluant. 200 mg of this material (0.28

mmol) and 32 mg of *N*-hydroxysuccinimide (0.28 mmol) were dissolved in 5 ml of CH_2Cl_2 and 57 mg of 1,3-dicyclohexylcarbodiimide (0.28 mmol) was added with stirring. The reaction was allowed to proceed for 1 h at room temperature after which the mixture was filtered to remove precipitate, and the solvent was removed by rotary evaporation yielding the activated lipid (**3**). A mixture of 5.6 mg of the peptide AcE4K (2.5 μmol), 4.1 mg of **3** (5.0 μmol) and 15 mg of triethylamine in 1 ml of dimethylsulf-oxide (DMSO) was heated to 65°C to achieve co-dissolution of the lipid and peptide and incubated for 1 h. After cooling, the lipopeptide (**4**) was precipitated by the addition of 5 ml of diethyl ether and cen-trifuged at $750 \times g$ for 5 min. The pellet was washed three times with 2 ml of diethyl ether repeating the centrifugation with each wash. The lipopeptide was dried under vacuum and its identity was confirmed by mass spectrometry. Purity as determined by pep-tide-to-lipid ratio using ^{1}H-NMR was found to be greater than 95%.

2.3. Preparation of liposomes

Chloroform solutions of lipids were dried by vor-tex mixing under nitrogen followed by the removal of residual solvent under high vacuum for 1 h. When lipopeptide was incorporated into the liposome prepa-rations, it was added to the dried lipids as a 1 mM solution in DMSO along with an equal volume of benzene/methanol (95:5) prior to freeze-drying for 5 h. Lipids were hydrated with appropriate buffers to concentrations ranging from 5 to 20 mM lipid. Five freeze-thaw cycles were used to ensure homogeneous mixture of the multilamellar vesicle (MLV) suspen-sions. The MLVs were extruded 10 times through two 100 nm pore-size polycarbonate filters (Costar, Cambridge, MA) in a high-pressure extruder (Lipex Biomembranes, Vancouver, BC) to produce large unilamellar vesicles (LUVs). Lipid concentrations were determined by phosphate assay as described by [15]. Depending on the lipid formulation, the mean diameter of the LUVs ranged from 100 to 135 nm as measured by quasi-elastic light scattering.

2.4. Circular dichroism

LUVs were prepared with 1% Lipo-AcE4K in POPC at a phospholipid concentration of 2.5 mM in

10 mM phosphate buffer adjusted to either pH 5.0 or pH 7.5. CD spectra from 200 to 250 nm were recorded on a Jasco J720 spectropolarimeter using a 1 mm quartz cuvette and accumulations of five scans. Under these conditions the CD spectra of the lipopeptide could be measured with minimal difficulties arising from absorbance and scattering due to the lipid. The spectra were corrected by subtracting control spectra of liposomes.

2.5. Tryptophan fluorescence

Tryptophan fluorescence spectra were recorded with an excitation wavelength of 280 nm over an emission range of 300–400 nm on a Perkin Elmer LS50 fluorometer using a 1 cm quartz cuvette thermostatted at 25°C. For the free peptide, 30 μl of 100 μM aqueous peptide solution was added to 2.97 ml of 10 mM phosphate buffer at pH 7.5 or pH 5.0, either with or without POPC LUVs (0.1 mM phospholipid, lipid/peptide ratio = 100). Spectra of lipopeptide incorporated into liposomes were obtained using the Lipo-AcE4K/POPC LUVs described above diluted to 0.1 mM POPC, 1.0 μM Lipo-AcE4K. The spectra were corrected by subtracting emission spectra of LUVs in phosphate buffer.

2.6. Preparation of erythrocyte membranes

Sealed erythrocyte ghosts were prepared by the method of [16]. Briefly, 4 ml of packed cells was washed 3 times in Hepes-buffered saline (HBS: 5 mM Hepes, 150 mM NaCl, pH 7.5). Washed cells were diluted 2-fold with HBS, lysed in 300 ml of 5 mM Hepes, 1 mM $MgSO_4$, pH 7.5, and pelleted at $20\,000 \times g$ for 20 min. Ghosts were resuspended in 200 ml of HBS containing 1 mM $MgCl_2$. The suspension was repelleted, washed twice more, and finally resuspended in 10 ml of HBS. Phospholipid concentration was determined by phosphate assay. The absence of glyceraldehyde-3-phosphate dehydrogenase activity [16] was used to confirm the formation of sealed right-side-out ghosts.

2.7. Lipid mixing fusion assays

The extent of membrane fusion as measured by lipid mixing in the presence of Lipo-AcE4K was monitored by the decrease in resonance energy trans-

fer (RET) resulting from dual fluorescent probe dilution [17]. LUVs of a desired lipid composition containing 0.5 mol% of both NBD-PE and Rh-PE were prepared in HMA buffer (10 mM Hepes, 10 mM Mes, 10 mM sodium acetate, 100 mM NaCl), pH 7.5. Lipo-AcE4K was either included in the lipid preparation as described above or added to labelled vesicles from a 2 mM solution in DMSO. Labelled vesicles were mixed with either unlabelled LUVs or erythrocyte ghosts in a lipid ratio of 1:3 at a total lipid concentration of 0.2 mM. Fluorescence was monitored at 25°C over 5 min with excitation at 465 nm, emission at 535 nm, and an emission cut-off filter at 530 nm. During the assays, 1 M HCl was added to decrease the pH to a desired value. HMA buffer has a linear pH response to acid volume over the pH range 4.0 to 7.5.

Each point in the lipid mixing timecourse was normalized by subtracting the fluorescence of a comparable assay lacking unlabelled vesicles (F_0) and dividing by the fluorescence achieved by infinite probe dilution determined by the addition of 25 μl of 100 mM Triton X-100 (F_{max}). Complete lipid mixing, as determined by a liposomes preparation with a total lipid composition corresponding to a 1:3 ratio of labelled to unlabelled POPC vesicles, gives a value of $\Delta F / \Delta F_{max}$ of approximately 80% under these conditions, and reported results have not been corrected by this factor.

2.8. Exchange of Lipo-AcE4K between membranes

POPC MLVs were prepared in HMA buffer at pH 7.5 as described above and pelleted at 12 000 rpm on a benchtop centrifuge at 5°C. The pellet was washed three times to ensure removal of any small lipid vesicles prior to determination of lipid concentration by phosphate assay. Thirty microlitres of POPC LUVs (10 mM lipid) was diluted in 1.09 ml of HMA buffer, pH 7.5, and 7.5 μl of 2 mM Lipo-AcE4K was added from DMSO stock. This preparation results in the incorporation of 10 mol% of the lipopeptide into the outer monolayer of the LUVs. After a 5 min pre-incubation at 25°C, 375 μl of 12.5 mM POPC MLVs were added as a sink for lipopeptide exchange. This 60-fold lipid excess represents approximately a 6-fold excess of lipid available for lipopeptide exchange, assuming 10% of the MLV lipid is exposed on the

outermost monolayer [18]. Following a 5 min incubation at 25°C, the MLVs were pelleted as above and the peptide and lipid content (phosphate assay) of the supernatant was determined. We used a micro-BCA assay kit as provided by Pierce (Rockford, IL) with the provided procedure to analyze for peptide. The results were compared to controls without MLVs or without Lipo-AcE4K.

2.9. Contents mixing and leakage

Liposomes of a desired composition were prepared containing 25 mM ANTS in HMA buffer, or 100 mM DPX in HMA buffer, or 6 mM ANTS plus 75 mM DPX (ANTS-DPX), at pH 7.5. External buffer was exchanged with HMA buffer on Sephadex G-25 columns prior to diluting to 10 mM lipid. To assay for contents mixing, 15 μl of the ANTS preparation and 45 μl of DPX liposomes were combined in 3 ml of HMA buffer. ANTS fluorescence was monitored over 5 min with the addition of 15 μl of 1 M HCl at 30 seconds to decrease the pH to 5.0. Excitation and emission wavelengths were 360 nm and 530 nm, respectively, and a 490 nm cut-off filter was used. Maximum quenching and zero leakage was determined by the fluorescence of the preparation containing both ANTS and DPX, and zero quenching was measured using only ANTS liposomes, both prior to the addition of acid. Leakage was quantified by comparing the maximum quenching result (0% leakage) with a similar assay to which 25 μl of 100 mM Triton X-100 was added (100% leakage).

2.10. Freeze-fracture electron microscopy

LUVs consisting of 10 mol% Lipo-AcE4K in EPC/Chol (55:45) were prepared in HMA buffer, pH 7.5, at a total lipid concentration of 5 mM. A sample at pH 5.0 was prepared by adding 1.5 μl of 1 M HCl to 100 μl of liposomes. After 5 min incubations at 25°C, samples at each pH were mixed 1:1 with glycerol and quickly frozen. Platinum/carbon replicas were prepared as described previously [19]. EPC/Chol (55:45) liposomes at pH 7.5 and 5.0 were used as controls.

2.11. Fluorescence microscopy

The dual-labelled LUVs containing 10 mol% Lipo-AcE4K in EPC/Chol (55:45) as described above for the lipid mixing assay were used. One hundred microlitres of 2.5 mM LUVs was added to 650 μl of HMA buffer, pH 7.5. After a 5 min pre-incubation, a 3-fold lipid excess of erythrocyte ghosts (250 μl of 3 mM lipid) was added. A 5 μl aliquot was removed prior to acidification with 15 μl of 1 M HCl, reducing the pH to 5.0. Samples at each pH were inspected by microscopy using both phase-contrast and fluorescence techniques.

3. Results

3.1. Solubilities of AcE4K and Lipo-AcE4K

The peptide AcE4K is soluble in aqueous solutions at pH 7.5 at concentrations up to 10 mM and highly soluble in DMSO. The use of the carboxy terminal lysine residue to couple the peptide to the lipid anchor, rather than the more commonly used cysteine-thioether chemistry, overcame earlier difficulties we had in purifying the corresponding C-terminal cysteine peptide. The lipopeptide Lipo-AcE4K was soluble only in DMSO and was added to assays from a 2 mM stock solution such that the amount of organic solvent was less than one percent by volume.

3.2. Circular dichroism and tryptophan fluorescence

The secondary structure of Lipo-AcE4K as a function of pH was determined by CD spectropolarimetry. In contrast to the free peptide which adopts a highly α-helical structure only at low pH in the presence of lipid vesicles (data not shown), the lipopeptide Lipo-AcE4K already exists in an α-helical conformation at pH 7.5 and the CD spectrum is not changed at pH 5.0 (Fig. 2). This result is surprising but does not provide information on the degree of interactions of the peptide with the lipid bilayer or its effects on membrane stability as a function of pH.

The fluorescence of tryptophan is dependent on the polarity of its environment. In an apolar environment, the emission maximum is shifted to lower wavelengths compared to that observed in aqueous media. This effect can be used to study the penetration of peptides bearing tryptophan residues into lipid bilayers [20]. The emission spectra for Lipo-AcE4K in POPC vesicles shown in Fig. 3 indicate that, even at pH 7.5, the tryptophan residue is somewhat protected from the aqueous medium having a λ_{max} of 340 nm
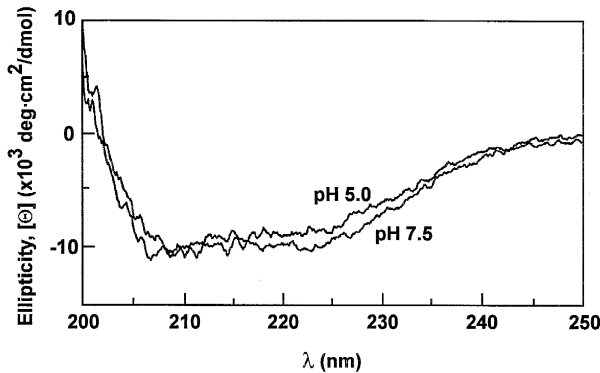
Fig. 2. Effect of pH on the secondary structure of Lipo-AcE4K. CD spectra of POPC LUVs (2.5 mM lipid) prepared with 1 mol% lipopeptide in phosphate buffer at pH 7.5 or pH 5.0. Spectra represent the average of 5 scans from which lipid signal has been subtracted.

compared to 355 nm for the free peptide. At pH 5.0, the λ_{max} is further reduced to 332 nm. This result suggests that, while no structural changes in the peptide were observed in the CD spectrum, Lipo-AcE4K penetrates further into the lipid bilayer upon neutralization of the acidic residues. However, the observed changes could also arise from the protection of the tryptophan residue from the aqueous medium through the formation of oligomeric complexes of the lipopeptide within the membrane.

3.3. Fusion of POPC LUVs induced by Lipo-AcE4K

The destabilization of membranes accompanying the observed changes in peptide structure and membrane penetration was studied by monitoring the fusion of lipid vesicles as measured by lipid mixing, contents mixing and leakage. Lipid mixing was monitored by the loss of RET between the fluorescently labelled lipids, NBD-PE and Rh-PE. Vesicles containing both probes are mixed with unlabelled vesicles, and membrane fusion results in probe dilution and increased NBD-PE fluorescence. Exchange of the labelled lipids does not occur over the duration of these experiments, even in highly aggregated systems [21], and fluorescence increases only upon mixing of membrane lipids.

Initially, we looked at fusion of POPC LUVs with 5 mol% Lipo-AcE4K added to the preformed vesicles from a DMSO stock solution. Lipid mixing fluorescence timecourses upon acidification to pH values between 7.0 and 4.0 are shown in Fig. 4. No lipid mixing was observed above pH 6.0. However, there was a substantial increase in mixing between pH 5.75 and 5.5. These changes may have physiological importance, in that the pH of the endosomal interior falls in the range of 5 to 6 [22]. At pH 5.0 an initial rapid increase in NBD-PE fluorescence levels of over 1–2 min, indicating a transient destabilization of the membrane. Further decreases in pH give even greater lipid mixing, and at pH 4.0 the initial rapid increase
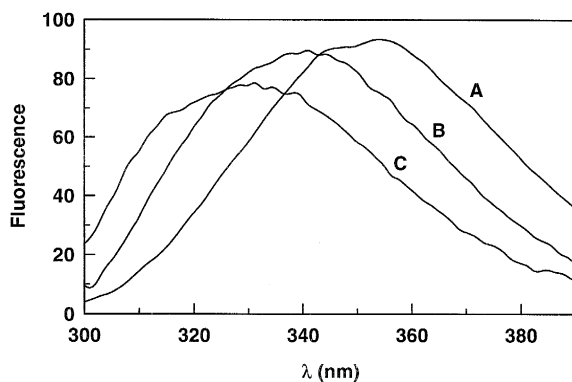

Fig. 3. Tryptophan fluorescence emission spectra of AcE4K and Lipo-AcE4K showing the effects lipid anchoring and of pH. POPC LUVs (0.1 mM lipid) were either incubated with 1 μM AcE4K at pH 7.5 (**A**) or prepared with 1 mol% Lipo-AcE4K in phosphate buffer at pH 7.5 (**B**) or pH 5.0 (**C**). Spectra were corrected by subtracting spectra of buffer or LUVs at the corresponding pH.
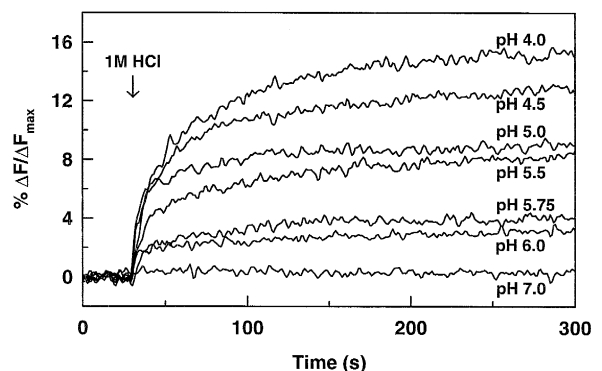

Fig. 4. Effect of pH on lipid mixing for 5 mol% Lipo-AcE4K in POPC LUVs. Lipopeptide was added from a 2 mM DMSO stock solution to a 1:3 mixture of labelled and unlabelled vesicles prior to the addition of 1M HCl to achieve the indicated pH.
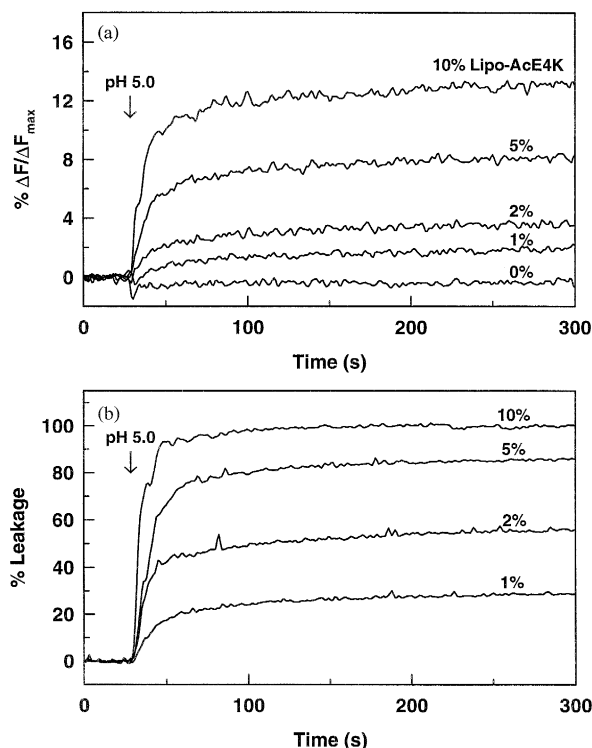
Fig. 5. Effects of Lipo-AcE4K concentration on lipid mixing and leakage in POPC LUVs. (a) Varying amounts of lipopeptide were added to a 1:3 mixture of labelled and unlabelled vesicles (0.2 mM total lipid) from a 2 mM stock solution in DMSO, such that the final DMSO concentration was less than 1% by volume. Lipid mixing assays were as described above, adding 1 M HCl at 30 s to achieve a final pH of 5.0. (b) Vesicles containing 6 mM ANTS and 75 mM DPX dissolved in HMA buffer, pH 7.5, were diluted to 0.2 mM lipid prior to addition of varying amounts of lipopeptide from a 2 mM DMSO stock solution. At 30 s, 1 M HCl was added to achieve a pH of 5.0.

changes not detectable by CD experiments or through the formation of oligomeric complexes.

To determine whether the pH-induced destabilization of membranes containing Lipo-AcE4K corresponded to fusion events with contents mixing in addition to the observed lipid mixing, we used the ANTS-DPX contents mixing assay [23]. Fusion between a liposome population containing the fluorescent marker ANTS and a second population containing the quencher DPX results in a loss of ANTS fluorescence. The assay is not affected by moderately acidic conditions and can distinguish contents mixing from probe leakage. Leakage was separately determined by monitoring ANTS dequenching for liposomes containing both ANTS and DPX.

For the range of Lipo-AcE4K concentrations used (1–10 mol%) in POPC vesicles, no contents mixing was observed. The ANTS-DPX assay revealed only the leakage of vesicle contents upon decreasing the pH to 5.0, and the extent of leakage observed corresponded to the concentration of lipopeptide as shown in Fig. 5(b). With 10 mol% Lipo-AcE4K in the outer monolayer, all of the probe was released within 1 min. At lower lipopeptide concentrations, most of the leakage occurred within the first minute followed by a much slower release of contents. This behaviour corresponds to the transient rapid lipid mixing which was observed, and again suggests a rapid loss of destabilizing capability for the lipopeptide.

To demonstrate fusion between LUVs bearing Lipo-AcE4K and either liposomes lacking lipopeptide or biological target membranes which are otherwise pH stable, it was first necessary to demonstrate that the lipopeptide does not exchange out of lipid bilayers into potential 'target' membranes. The transfer of Lipo-AcE4K was investigated by incubating POPC LUVs containing 10 mol% Lipo-AcE4K added after vesicle formation with a large excess of POPC MLVs. The MLVs were separated from LUVs by centrifugation. A comparison of the peptide contents of the Lipo-AcE4K-bearing LUVs before and after incubation with MLVs is given in Fig. 6(a). It is clear that there is no exchange of Lipo-AcE4K out of the LUV population when incubated with MLVs.

As seen in Fig. 6(b), very little lipid mixing is observed when only one liposome population contains 5 mol% Lipo-AcE4K compared to the level attained when the lipopeptide is present in both mem-

in fluorescence is followed by a slower rise over several minutes.

The effect of Lipo-AcE4K concentration in the outer monolayer of POPC vesicles on the degree of lipid mixing at pH 5.0 is shown in Fig. 5(a). Small but significant increases in NBD-PE fluorescence were observed with as little as 1 mol% Lipo-AcE4K, and the level of mixing achieved increased with lipopeptide concentration up to 10 mol%, the maximum level assayed. In all cases, the observed increase in fluorescence was arrested within 1 or 2 min. The transient nature of the lipid mixing in all of these cases suggests the loss of destabilizing capability of the lipopeptide, perhaps through conformational
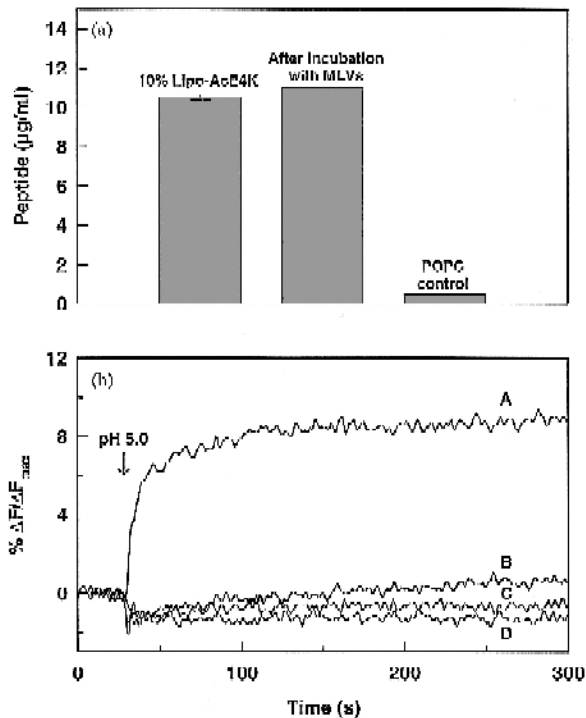
Fig. 6. Exchange of Lipo-AcE4K between vesicle populations and lipid mixing with membranes lacking lipopeptide. (a) 10 mol% Lipo-AcE4K was added to prepared POPC LUVs, and the amount of peptide associated with POPC vesicles was determined by micro-BCA assay before and after incubation with POPC MLVs. A control experiment using LUVs without lipopeptide is also shown. Assays were carried out in duplicate, and deviations from means were negligible except where error bars are shown. (b) Lipid mixing assays were performed after pre-incubation of selected liposome populations with sufficient Lipo-AcE4K to achieve a 5 mol% concentration in liposomal outer monolayers. The lipopeptide was included in both fluorescently labelled and unlabelled populations (**A**), in labelled vesicles only (**B**), in unlabelled vesicles only (**C**), or in neither labelled nor unlabelled liposomes (**D**).

branes. There was only a small difference in this result when the Lipo-AcE4K was incorporated into the fluorescently labelled population rather the unlabelled population, reflecting the probability differences resulting from the mixing ratio of 1:3 labelled to unlabelled. It appears that, while Lipo-AcE4K can promote lipid mixing in POPC LUVs, it must be present in both vesicle populations to do so.

3.4. Fusion and leakage in EPC / Chol vesicles

In order to achieve higher levels of membrane fusion, we have looked at the effect of Lipo-AcE4K

on the stability of EPC/Chol (55:45) LUVs which more closely approximate the lipid composition of biological membranes. EPC is a naturally occurring mixture of phosphatidylcholine species bearing a variety of fatty acyl chains, and it consists predominantly of POPC. While the addition of cholesterol to phospholipid bilayers decreases membrane permeability by effecting tighter packing lipids, cholesterol can also promote membrane fusion by inducing the formation of non-bilayer lipid phases [24].

As shown in Fig. 7(a), Lipo-AcE4K at a concentration of 5 or 10 mol% is more effective at promoting lipid mixing in EPC/Chol (55:45) LUVs than in either EPC or POPC LUVs (cf. Fig. 5(a)). Again a transient rapid increase in fluorescence is observed; however, higher levels of fluorescence are achieved and lipid mixing continues at a reduced rate for the
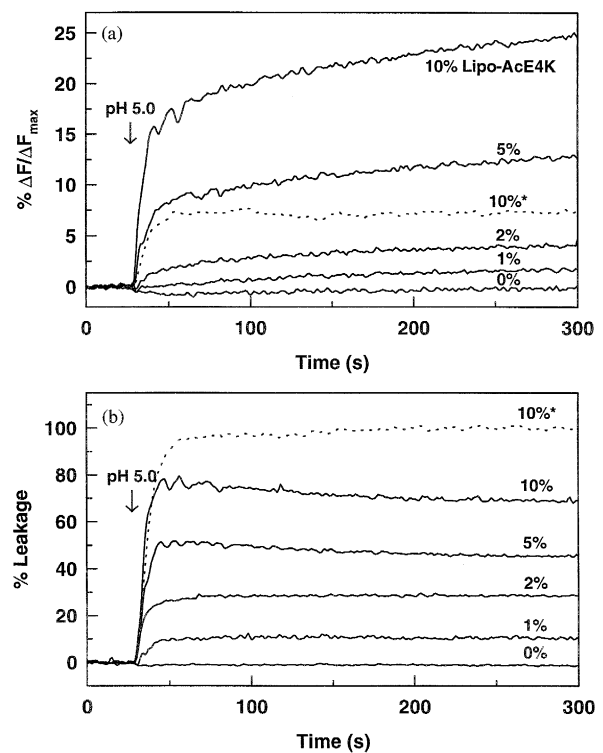

Fig. 7. Lipid mixing and leakage in EPC/Chol (55:45) LUVs. (a) 0 to 10 mol% Lipo-AcE4K was added to a 1:3 mixture of labelled and unlabelled vesicles (0.2 mM total lipid) from a 2 mM stock solution in DMSO. Lipid mixing assays were as described above, adding 1 M HCl at 30 s to achieve a final pH of 5.0. (b) Leakage assays for 0 to 10 mol% Lipo-AcE4K added to liposomes containing 6 mM ANTS and 75 mM DPX. [*] For comparison, corresponding assays for 10 mol% Lipo-AcE4K in EPC LUVs are shown (dotted lines).
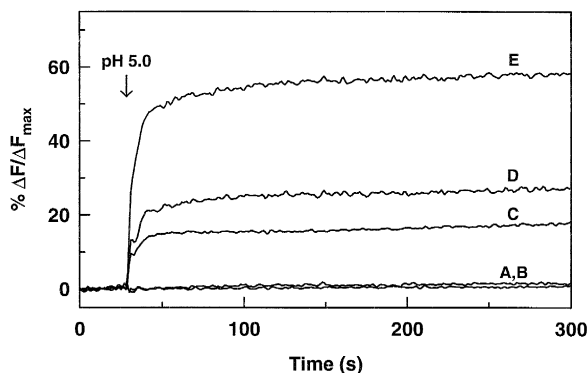
Fig. 8. Effect of transbilayer distribution of Lipo-AcE4K on lipid mixing in EPC/Chol (55:45) LUVs. 10 mol% Lipo-AcE4K was either present in the outer monolayer of liposomes (**B**, **C**) or in both monolayers (**D**, **E**) and was present in only the fluorescently labelled LUV population (**B**, **D**) or in both labelled and unlabelled LUVs (**C**, **E**). A control lipid mixing assay where neither vesicle population contained lipopeptide is also shown (**A**).

duration of the assay. Interestingly, the corresponding ANTS-DPX leakage results for EPC/Chol liposomes, given in Fig. 7(b), indicate lower levels of leakage at all concentrations of Lipo-AcE4K than were observed for POPC (Fig. 5(b)) or EPC (only 10 mol% Lipo-AcE4K data shown). However, leakage of vesicle contents remains substantial, and in no case was contents mixing of vesicles observed.

3.5. Effects of transbilayer distribution of Lipo-AcE4K

Increased levels of lipid mixing were observed in EPC/Chol (55:45) vesicles when Lipo-AcE4K was present on both the inner and outer monolayers of the liposomes. This was achieved by adding the lipopeptide to the lipid preparation prior to freeze-drying, hydration, and extrusion. As shown in Fig. 8, lipid mixing between LUVs containing 10 mol% Lipo-
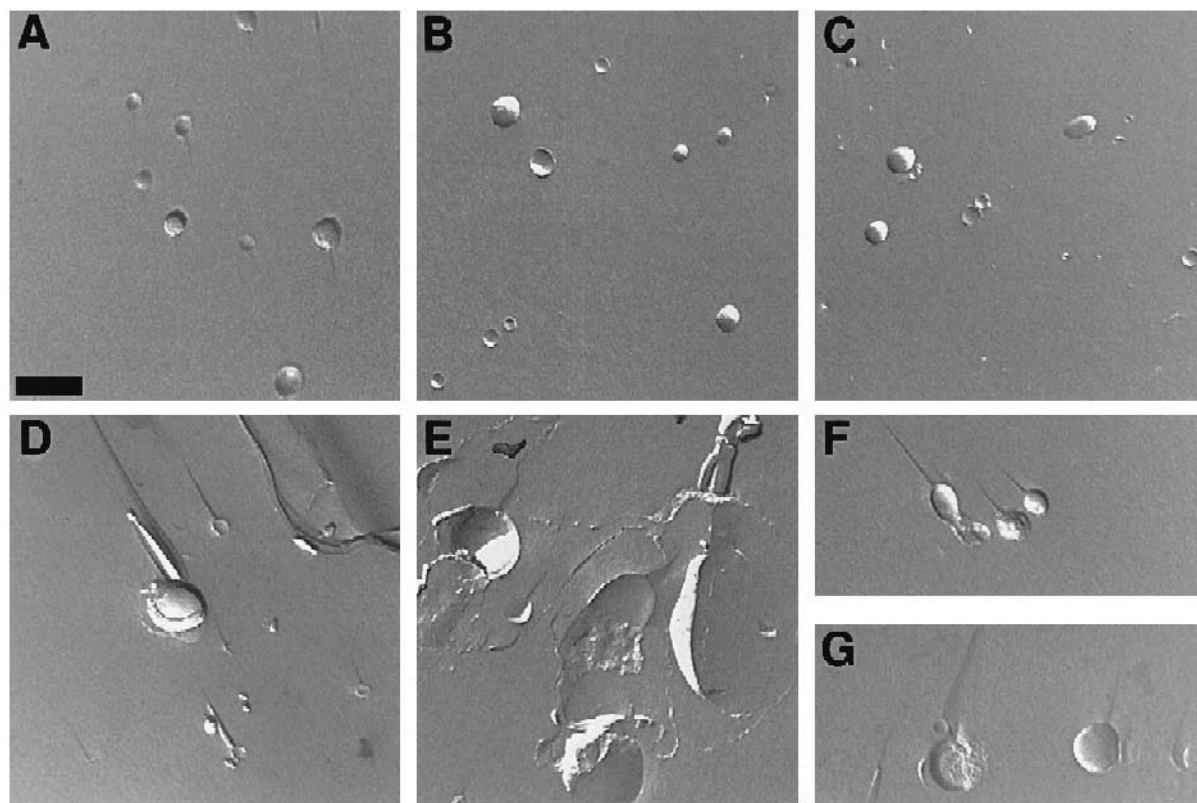

Fig. 9. Freeze-fracture electron micrographs of EPC/Chol liposomes: effect of Lipo-AcE4K and pH. EPC/Chol (55:45) LUVs were prepared with (**C**, **D–G**) and without (**A**, **B**) 10 mol% Lipo-AcE4K at a total lipid concentration of 5 mM in HMA buffer. Platinum-carbon replicas were prepared at pH 7.5 (**A**, **C**) or 5 min following acidification to pH 5.0 by the addition of 1 M HCl (**B**, **D–G**). Original magnification was 20 000 ×, and bar the represents 200 nm.

AcE4K prepared by this method gave values of $\Delta F / \Delta F_{max}$ approaching 60% at 5 min. Furthermore, mixing these LUVs and an EPC/Chol (55:45) preparation lacking lipopeptide also resulted in substantial membrane fusion. In contrast, LUVs prepared as before with 10 mol% Lipo-AcE4K only on the outer monolayer could fuse with themselves, but not with EPC/Chol LUVs.

3.6. Freeze-fracture electron microscopy

Freeze-fracture micrographs of EPC/Chol (55:45) LUVs bearing 10 mol% Lipo-AcE4K on both inner and outer membranes are shown in Fig. 9. Samples without Lipo-AcE4K or those containing lipopeptide at pH 7.5 gave smooth fracture surfaces and had size distributions typical of those observed for LUVs extruded through 100 nm filters. Samples with Lipo-AcE4K acidified to pH 5.0, however, exhibited numerous larger lipid structures, indicating fusion of liposomes. In addition, many of these larger structures exhibited rough surfaces which could arise from penetration of the peptide portion of Lipo-AcE4K into the membrane. Furthermore, many of these vesicles were cross-fractured, suggesting that the preferential cleavage between the inner and outer monolayers of the lipid bilayer is disrupted by the presence of the lipopeptide.

3.7. Lipid mixing with erythrocyte ghosts

Ultimately, we are interested in the destabilizing properties of Lipo-AcE4K directed toward biological membranes. As a model for such systems, we examined lipid mixing between EPC/Chol (55:45) liposomes containing Lipo-AcE4K with erythrocyte ghosts. The ghost preparation used here included 1 mM $MgSO_4$ in the lysis and washing buffers as described by Steck and Kant [16]. Analysis for glycerol-3-phosphate dehydrogenase activity confirmed the formation of sealed, right-side-out ghosts which was not the case for a preparation without 1 mM $MgSO_4$ (data not shown).

Fluorescence lipid mixing assays with EPC/Chol and erythrocyte ghosts were performed using two different liposome preparations. First, Lipo-AcE4K was added to EPC/Chol (55:45) LUVs from a DMSO
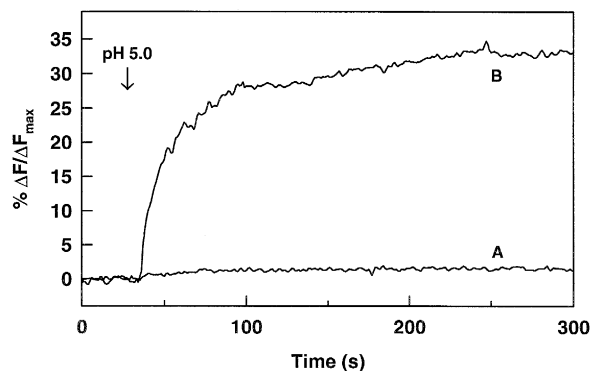

Fig. 10. Effect of method of lipopeptide incorporation into EPC/Chol (55:45) LUVs on lipid mixing with erythrocyte membranes. (**A**) 10 mol% Lipo-AcE4K was added from DMSO stock solution to fluorescently labelled liposomes and pre-incubated at 25°C for 5 min prior to addition of erythrocyte membranes. The lipopeptide is present only in the outer monolayer of vesicles. (**B**) Lipid-mixing assay of co-lyophilized 10 mol% Lipo-AcE4K in EPC/Chol (55:45) with erythrocyte ghosts. The lipopeptide is present in inner and outer monolayers of the fluorescently labelled vesicles.

stock solution leading to incorporation of the lipopeptide into the outer monolayers of the LUVs at a concentration of 10 mol% relative to surface-exposed lipid. Addition of erythrocyte ghosts to this preparation gave limited lipid mixing ($\Delta F / \Delta F_{max} \sim 2\%$) when the pH was decreased to 5.0 (Fig. 10). This result is in agreement with that shown in Figs. 7 and 9 in which incorporation of Lipo-AcE4K into the outer monolayer of one population of vesicles was insufficient to give substantial lipid mixing with a second population of membranes. A second liposome preparation with 10 mol% Lipo-AcE4K in EPC/Chol (55:45), incorporating lipopeptide into both inner and outer monolayers, gave much higher levels of lipid mixing with erythrocyte ghosts at pH 5.0, $\Delta F / \Delta F_{max}$ values approaching 40% at 5 min.

Further evidence for lipid mixing with erythrocyte ghosts was demonstrated by fluorescence microscopy. The fluorescently labelled liposome preparations from the lipid-mixing assays were also used for this procedure. Rh-PE is present in these preparations at a self-quenching concentration [25]. This permits the detection of lipid mixing between labelled liposomes and erythrocyte ghosts as an increase in Rh-PE fluorescence upon dilution into the target. Fig. 11 illustrates the effect of decreasing the

pH for a mixture of LUVs prepared from EPC/Chol (55:45) co-lyophilized with 10 mol% Lipo-AcE4K and erythrocyte ghosts. Phase contrast micrographs are shown with corresponding fluorescence images at pH 7.5 and 5.0. While the appearance of Rh-PE fluorescence in the erythrocyte membranes is the most striking effect of lowering the pH, aggregation of the erythrocyte ghosts is also apparent. Labelled EPC/Chol (55:45) LUVs without lipopeptide gave no lipid mixing or aggregation with erythrocyte ghosts at pH 5.0 (not shown).

4. Discussion

Coupling the peptide to a lipid anchor and incorporating it into liposomes affects both the structure adopted by the peptide portion and the extent of its
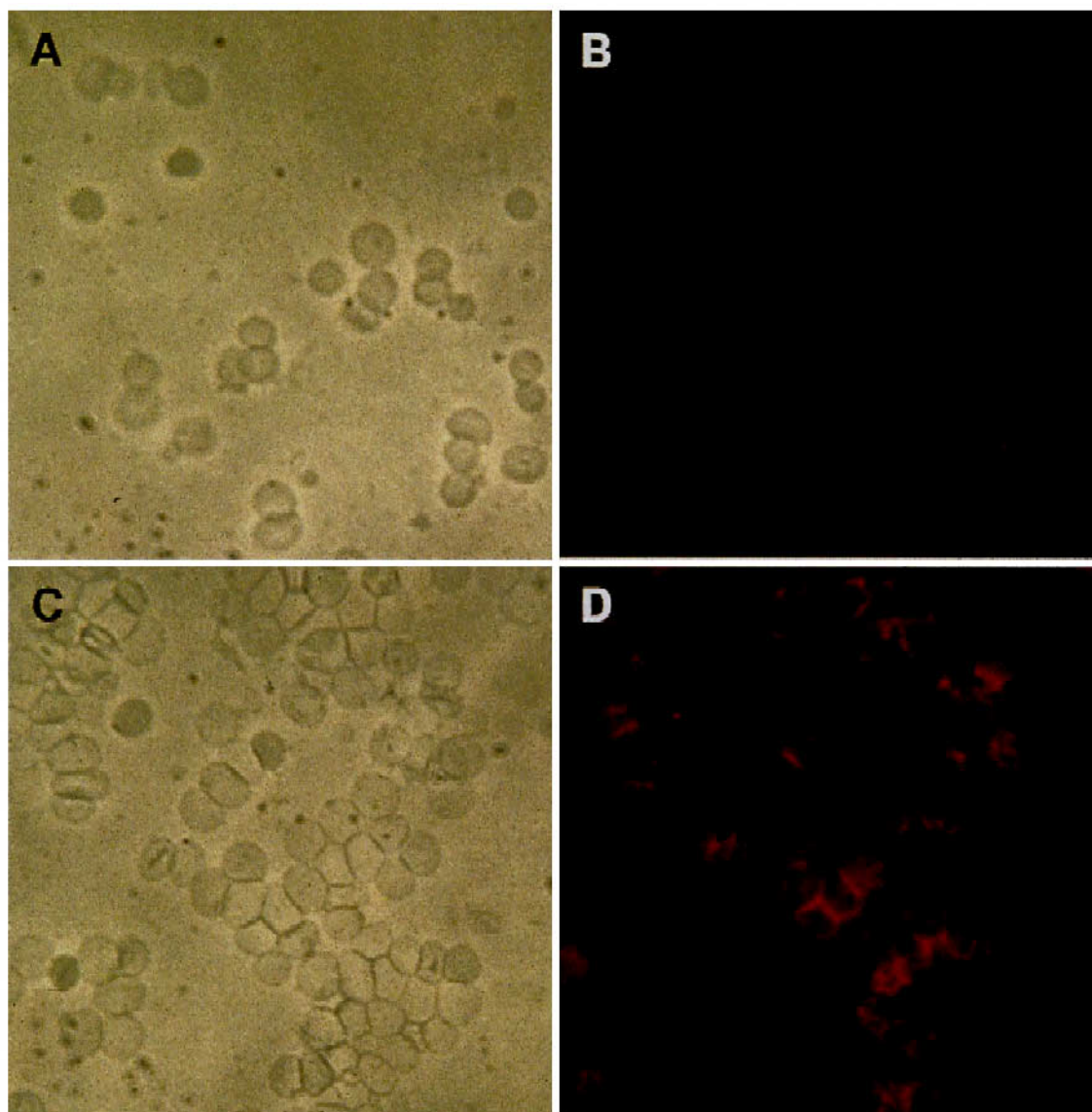

Fig. 11. Fluorescence micrographs showing the appearance of Rh-PE in erythrocyte membranes upon lipid mixing with 10 mol% Lipo-AcE4K in EPC/Chol (55:45). Liposomes were prepared from a co-lyophilized preparation of 10 mol% Lipo-AcE4K in EPC/Chol containing 0.5 mol% each of NBD-PE and Rh-PE. Liposome and erythrocyte membranes were mixed in a 1:3 lipid ratio (1 mM total lipid): (A) phase contrast and (B) Rh-PE fluorescence at pH 7.5; (C) phase contrast and (D) Rh-PE fluorescence after reducing pH to 5.0.

interactions with the lipid bilayer as a function of pH. The CD spectra of Lipo-AcE4K reveal that the anchored peptide adopts an α-helical structure, even at neutral pH. Presumably this occurs as a result of the imposed proximity of the membrane surface and hydrophobic interactions of the peptide with the lipid bilayer. There is no evidence of further structural changes upon neutralization of acidic residues. However, the tryptophan fluorescence maxima suggest that the anchored peptide experiences a more hydrophobic environment upon acidification of the medium compared to its uncoupled counterpart. Based on the lipid-mixing and electron microscopy results presented here, we believe that this hydrophobic environment results from increased penetration of Lipo-AcE4K into the lipid bilayer and not simply from a loss of charge on neighbouring residues or the formation of lipopeptide complexes.

The extent of destabilization induced by Lipo-AcE4K depends not only on pH and lipopeptide concentration, but also on membrane lipid composition and on the transbilayer distribution of the lipopeptide. When Lipo-AcE4K was added to pre-formed vesicles, lipid mixing was only substantial between vesicle populations that each contained the lipopeptide. This suggests that when Lipo-AcE4K is incorporated into the outer membranes of lipid vesicles in this way, it can only penetrate and destabilize the membrane in which it is anchored. Alternatively, if it inserts into the membranes of target vesicles, it does not destabilize the target vesicles sufficiently to promote lipid mixing between the two populations. In caution it should be noted that fusion within the labelled population of lipopeptide-bearing vesicles which cannot be detected by the assay may simply be the predominant process and that lipid mixing with a second population would be promoted under more constrained circumstances, e.g., within an endosome.

Our results are very different from those previously reported for HA-derived peptides lacking membrane anchors. Düzgünes and Shavnin [6] used a 17 amino acid peptide from the N-terminus of HA2 X31 wild-type sequence and found that it gave extensive leakage for EPC LUVs at both neutral and low pH and no lipid mixing under any conditions. Rafalski et al. [10] reported pH-dependent leakage for the 20 amino acid wt and for E4, but no lipid mixing in either POPC or POPC/Chol vesicles. It is clear that

features such as peptide length, acidity, and membrane-anchoring all influence the membrane-destabilizing ability of these peptides.

The absence of contents mixing, the occurrence of rapid, transient lipid mixing and the accompanying transient leakage observed in the present system clearly do not describe the fusion process as achieved by the virus. The leakage results are consistent with previous studies on the destabilization of lipid vesicles with viral fusion peptides. To date, contents mixing arising from a non-leaky fusion event induced by a fusion peptide has not been convincingly demonstrated. However, the ability of a fusion peptide to destabilize lipid bilayers may represent its functional role in destabilizing target cellular membranes, while the entire fusion protein is required to achieve a complete fusion event.

In summary, the lipopeptide Lipo-AcE4K forms stable bilayers in POPC and EPC/Chol LUVs at concentrations up to 10 mol% at pH 7.5. Destabilization of these lipid vesicles can be induced by decreasing the pH below 6.0 which corresponds to the conditions under which the viral protein, influenza HA from which it is derived causes membrane fusion. This membrane destabilization not only results in extensive leakage of liposomal contents, as has been demonstrated with a variety of other viral fusion peptides and synthetic amphipathic helices, but also in lipid mixing of LUVs as determined by fluorescent lipid probe dilution, and coalescence of lipid membranes shown by freeze-fracture electron microscopy. The extent of lipid mixing depends on pH, membrane composition, and the concentration of the lipopeptide as well as on its distribution between the membrane leaflets. Incorporation of Lipo-AcE4K into both inner and outer leaflets of EPC/Chol (55:45) LUVs causes these vesicles to fuse with liposomes lacking Lipo-AcE4K and with erythrocyte ghosts at low pH. This is the first example of fusion induced by a membrane-anchored fusion peptide with a biological membrane.

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