The N-terminal segment of pulmonary surfactant lipopeptide SP-C has intrinsic propensity to interact with and perturb phospholipid bilayers

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In the present study, 13-residue peptides with sequences corresponding to the native N-terminal segment of pulmonary SP-C (surfactant protein C) have been synthesized and their interaction with phospholipid bilayers characterized. The peptides are soluble in aqueous media but associate spontaneously with bilayers composed of either zwitterionic (phosphatidylcholine) or anionic (phosphatidylglycerol) phospholipids. The peptides show higher affinity for anionic than for zwitterionic membranes. Interaction of the peptides with both zwitterionic and anionic membranes promotes phospholipid vesicle aggregation, and leakage of the aqueous content of the vesicles. The lipid–peptide interaction includes a significant hydrophobic component for both zwitterionic and anionic membranes, although the interaction with phosphatidylglycerol bilayers is also electrostatic in nature. The effects

of the SP-C N-terminal peptides on the membrane structure are mediated by significant perturbations of the packing order and mobility of phospholipid acyl chain segments deep in the bilayer, as detected by differential scanning calorimetry and spin-label ESR. These results suggest that the N-terminal region of SP-C, even in the absence of acylation, possesses an intrinsic propensity to interact with and perturb phospholipid bilayers, thereby potentially facilitating SP-C promoting bilayer-monolayer transitions at the alveolar spaces.

Key words: lipid–protein interaction, membrane perturbation, monolayer, phospholipid bilayer, protein palmitoylation, pulmonary surfactant.

INTRODUCTION

Lipopeptide SP-C (surfactant protein C) is expressed specifically in mammalian lung tissue, forming part of pulmonary surfactant, a lipid–protein complex that modulates surface tension at the respiratory air–liquid interface to stabilize bronchoalveolar structure. Phospholipids, especially DPPC (1,2-dipalmitoylphosphatidylcholine), play a major biophysical role in surfactant because of their ability to form stable interfacial monomolecular films, which are able to achieve and sustain very low surface tensions. Other components of the surfactant complex, such as the anionic phospholipid PG (1,2-diacylphosphatidylglycerol) and specific surfactant-associated proteins, are required for pulmonary surfactant to undergo complex and dynamic transformations in the airspaces [1,2]. Hydrophobic SP-B (surfactant protein B) and SP-C are essential to promote transfer of surface-active complexes to the interface, and to modulate the physical properties of the surfactant film under the dynamic conditions imposed by the respiratory cycle [3,4]. The precise role of SP-C in respiratory physiology has still to be defined. This protein is not strictly required to initiate respiratory function at birth, as seen from animals in which expression of the SP-C gene has been genetically deactivated [5,6]. However, surfactant obtained from the lungs of these SP-C-knockout animals seem to be intrinsically unstable at low lung volumes. Consistent with this fact, genetic deficiencies in the structure and expression of the SP-C gene have been found in patients developing chronic respiratory diseases [7,8].

SP-C is a 35-amino acid lipopeptide with a molecular mass of 4 kDa [9]. Its structure has been determined in chloroform/methanol solutions [10]. The protein consists of a regular hydrophobic α -helix comprising 23 residues, and a more polar 10-residue N-terminal segment that contains stoichiometrically palmitoylated cysteines which has no defined conformation in organic solvents or in detergent micelles [10,11]. The C-terminal segment adopts a transmembrane orientation in phospholipid bilayers [12,13] but the disposition of the N-terminal segment in membranes is unknown. Numerous studies have characterized lipid–protein interactions and lipid-perturbing effects of SP-C, in an attempt to understand the ability of SP-C to modulate the surface-active properties of surfactant in molecular terms [13–21]. A major problem is that most of the physico-chemical features of SP-C are dominated by contributions from the hydrophobic α -helix, making it difficult to assign relevant properties originating from the N-terminal segment of the protein. Lipid–protein interactions involving this region of SP-C have been studied previously by using protein forms that were chemically derivatized to introduce extrinsic probes at the N-terminal end [22,23]. Those studies indicated that the N-terminal segment of SP-C is located at the surface of phospholipid bilayers, exposed to the aqueous environment. The presence of anionic lipid species is found to have important effects on the disposition of the N-terminal segment of SP-C in membranes. The main objective of the present work was to test whether the primary sequence of the N-terminal segment of SP-C has intrinsic determinants to cause

Abbreviations used: ANTS, 8-aminonaphthalene-1,3,6-trisulphonic acid; Br_(6,7)-PC, 1-palmitoyl-2-stearoyl(6-7)dibromo-*sn*-glycerol-3-phosphocholine; Br_(11,12)-PC, 1-palmitoyl-2-stearoyl(11-12)dibromo-*sn*-glycerol-3-phosphocholine; DPPC, 1,2-dipalmitoylphosphatidylcholine; DPPG, 1,2-dipalmitoylphosphatidylglycerol; DPX, *N,N'*-*p*-xylene-bis-pyrimidinium bromide; PC, 1,2-diacylphosphatidylcholine; PG, 1,2-diacylphosphatidylglycerol; SP-B, surfactant protein B; SP-C, surfactant protein C; LPS, lipopolysaccharide; 5-PCSL, 1-acyl-2-[5-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycerol-3-phosphocholine; 5-PGSL, 1-acyl-2-[5-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]-phosphatidylglycerol.

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it to associate with and perturb membranes, independently of its acylation state. We propose that the intrinsic properties of this region in the native protein, especially its ability to interact with and perturb phospholipid surfaces, are relevant to understanding the role of SP-C in pulmonary surfactant function.

EXPERIMENTAL

Materials

Chloroform and methanol were HPLC-grade solvents from Scharlau (Barcelona, Spain). The lipids DPPC, DPPG (1,2-dipalmitoylphosphatidylglycerol), egg yolk PC (1,2-diacylphosphatidylcholine) and egg yolk PG (produced by transphosphatidylation of egg yolk PC), Br_(6,7)-PC [1-palmitoyl-2-stearoyl (6-7)dibromo-*sn*-glycerol-3-phosphocholine] and Br_(11,12)-PC [1-palmitoyl-2-stearoyl(11-12)dibromo-*sn*-glycerol-3-phosphocholine] were purchased from Avanti Polar Lipids (Birmingham, AL, U.S.A.). Radiolabelled 1,2-di-[1-¹⁴C]palmitoyl-PC (112 Ci/mol) was from Amersham International (Little Chalfont, Bucks., U.K.). Spin-labelled phosphatidylcholines, with the nitroxide group at different positions of the *sn*-2 acyl chain, were synthesized as described by Marsh and Watts [24]. The spin labels were stored at -20 °C in chloroform/methanol (2/1, v/v) solutions at a concentration of 1 mg/ml.

Peptide synthesis and purification

Two 13-residue peptides were synthesized by Fmoc (fluorenylmethoxycarbonyl) chemistry, as described elsewhere [25], taking the sequence of porcine SP-C as a template. The N-terminal leucine residue in the native sequence was replaced in the two synthetic peptides by tryptophan, with the aim of using this as an intrinsic probe to characterize lipid-protein interactions by fluorescence spectroscopy. A peptide, named pSP-C_{L1W} (NH₂-WRI-PCCPVNLKRL-CONH₂), has the two cysteines of the native sequence bearing free thiol groups, while the peptide pSP-C_{L1Wb} (NH₂-WRIPOOPVNLKRL-CONH₂) is a variant with the two thiols blocked by acetamidomethyl groups (O). Purity of the products of synthesis was checked by MALDI-TOF (matrix-assisted laser desorption-ionization time-of-flight) MS analysis and N-terminal Edman amino acid sequencing.

Lipid/peptide samples

Phospholipid vesicles for the binding experiments were prepared by extrusion of the appropriate lipid suspensions, equilibrated at temperatures above the chain-melting temperature of the corresponding lipid, through 0.1 µm (pore diameter) polycarbonate membranes (Nucleopore; Costar, Cambridge, MA, U.S.A.) in an Extruder (Lipex, Biomembranes, Vancouver, Canada). The lipid suspensions contained a trace of radioactive phospholipid (1,2-di-[¹⁴C]palmitoyl-PC, 55 mCi/mol) to facilitate rapid lipid quantitation. The extruded vesicles (phospholipid concentration, 0.43 mM) were incubated overnight at 37 °C, with or without peptide injected from a concentrated methanolic solution (17 µg/ml final concentration, 1:44 peptide/lipid molar ratio).

In the fluorescence spectroscopy experiments, small aliquots, typically 5–10 µl, of concentrated 1 mg/ml methanolic solutions of the peptides were injected into suspensions of lipid vesicles prepared by extrusion as described above in 50 mM Hepes buffer containing 150 mM NaCl, pH 7.0. To analyse quenching of peptide tryptophan fluorescence by brominated lipids, PC vesicles were prepared containing different weight percentages of Br_(6,7)-

PC or Br_(11,12)-PC, with or without peptide, at a peptide/lipid ratio of 1:45 (mol/mol).

To carry out differential scanning calorimetry experiments, DPPC and the desired amount of peptide (stored in methanol solution) were pre-mixed in chloroform/methanol (2:1, v/v), dried under a N₂ stream, and reconstituted as multilamellar suspensions. To achieve this, lipid or peptide/lipid samples were hydrated in 1 ml of buffer (50 mM Hepes/150 mM NaCl, pH 7) at 50 °C for 1 h, with occasional vortex mixing.

For ESR experiments, appropriate amounts of DPPC or DPPG and the selected spin-labelled lipid (1 mol %) were dissolved in chloroform/methanol (2:1, v/v), and mixed with the desired amount of peptide. Multilamellar suspensions were prepared in 100 µl of buffer (50 mM Hepes/150 mM NaCl, pH 7), and the reconstituted material was pelleted in 100 µl capillary tubes for ESR spectroscopy by centrifugation at 1500 g in a bench-top centrifuge.

Lipid/peptide-binding experiments

Free and vesicle-bound peptide fractions were separated by centrifugation of the peptide-lipid mixtures, after incubation under the conditions described above, in a Beckman Airfuge (rotor A95) for 1 h at 130 000 g at 4 °C. After centrifugation, 80 µl fractions were taken from top to bottom of each tube and the tryptophan fluorescence emission spectrum was recorded from each fraction as explained below. Peptide concentration was determined by amino acid analysis, and the phospholipid concentration by quantification of radioactivity.

Fluorescence spectroscopy

Tryptophan fluorescence emission spectra of peptides were obtained, using 280 nm as the excitation wavelength, either in a Perkin-Elmer MPF-44E or in a SLM-Aminco AB-2 spectrofluorimeter, operated in the ratio mode [26]. The spectra were recorded at 25 °C at a scan rate of 1 nm/s, in cells under thermostat-controlled conditions with a 0.2 cm optical path. The slit widths were 5 nm for both excitation and emission. All spectra were corrected for scattering and inner filter effect as described previously [26].

Phospholipid vesicle aggregation assay

Peptide-induced phospholipid vesicle aggregation assays were performed at 25 or 45 °C in a Beckman DU-640 spectrophotometer, by monitoring changes in apparent absorbance at 400 nm caused by increase in light scattering. Phospholipid vesicles were added to both the sample and the reference cuvettes, in a total volume of 300 µl of buffer (50 mM Hepes, pH 7, containing 150 mM NaCl). After 10 min of equilibration, different amounts of peptide were added to the sample cuvette from a concentrated methanolic solution, and the changes in absorbance at 400 nm were monitored at 1 min intervals over 30 min. As a control, an identical amount of methanol was added to a sample of vesicles alone.

Leakage of vesicle aqueous contents

Peptide-induced release of aqueous vesicle content was measured by using the ANTS (8-aminonaphthalene-1,3,6-trisulphonic acid)/DPX (*N,N'*-*p*-xylene-bis-pyrimidinium bromide) assay [27]. DPPC or PG vesicles were prepared by extrusion of lipid suspensions prepared in 50 mM Hepes buffer, pH 7, containing 30 mM NaCl, 12.5 mM of the fluorophore ANTS and 45 mM DPX as a quencher. Vesicles were separated from unencapsulated

material on a Sephadex G-75 column (Amersham Biosciences, Uppsala, Sweden) by using 50 mM Hepes, pH 7, containing 150 mM NaCl, as elution buffer. We were not successful in preparing stable DPPG vesicles that encapsulated significant amounts of fluorophore. In a typical assay, a given volume of peptide was added from a concentrated methanolic solution to the ANTS/DPX-loaded vesicles at 75 μ M lipid. The resulting leakage was then followed by measuring the increase in fluorescence emission intensity at 536 nm, upon excitation at 386 nm, in a SLM-Aminco AB-2 spectrofluorimeter. The 0 and 100 % limits of leakage were taken as the fluorescence intensity of the DPPC or PG vesicle suspension before and after, respectively, addition of an aliquot of Triton X-100 to yield a final detergent concentration of 0.5 % (w/v).

Differential scanning calorimetry

Multilamellar suspensions of DPPC (1 mg/ml, final concentration), in the absence or in the presence of different amounts of peptide, were loaded in the sample cell of a MicroCal MC-2 microcalorimeter, with buffer in the reference cell. Three calorimetric scans between 25 and 60 °C were collected from each sample, at a rate of 0.5 °C/min.

ESR spectroscopy

ESR spectra of lipid and lipid/peptide samples were recorded on a Varian E-12 Century Line 9 GHz spectrometer equipped with a nitrogen gas flow temperature-regulation system. The sealed capillaries (1 mm outer diameter) were placed in a quartz tube containing light silicone oil for thermal stability. Temperature was measured with a fine-wire thermocouple positioned in the silicone oil at the top of the microwave cavity. Spectra were collected digitally on a personal computer with Labmaster interface. Instrumental settings were as follows: 10 mW microwave power, 1.25 G modulation amplitude, 100 kHz modulation frequency, 0.25 s time constant, 4 min scan time, 100 G scan range and 3245 G centre field. Several scans, typically three to five, were accumulated to improve the signal-to-noise ratio. See Perez-Gil et al. [18] for details of spectral analysis.

RESULTS

Lipid/peptide binding

Figure 1 illustrates the ability of peptides pSP-C_{L1W} and pSP-C_{L1Wb} to distribute between water and the lipid phase in a suspension of PC or PG vesicles. In the absence of lipids, the two peptides were soluble in the buffer and no peptide accumulation was detected at the bottom of the tube after extensive centrifugation. When PC or PG vesicle suspensions were centrifuged, more than 90 % of the lipid was collected in the pellet. If lipid vesicle suspensions were incubated with either pSP-C_{L1W} or pSP-C_{L1Wb} peptide, the lipids were again completely pelleted upon centrifugation, but now substantial portions of both peptides were co-pelleted with the vesicles. PC membranes bound around 70 % of either peptide, while PG vesicles bound more than 90 % of pSP-C_{L1Wb} or pSP-C_{L1W}. Determination of the proportion of either peptide bound to the membranes allows estimation of their apparent mole fraction surface partition coefficients K [28], according to the equation:

$$f_{\text{bound}} = KL/(W + KL) \quad (1)$$

where f_{bound} is the molar fraction of peptide bound to the mem-

branes, K is the mole-fraction partition coefficient, L is the concentration of accessible lipid (60 % of the total lipid concentration, representing the outer leaflet of the membrane bilayers) and W is the molar concentration of water (55.3 M at 25 °C). The blocked peptide pSP-C_{L1Wb} had a partition coefficient K of $\approx 4.6 \times 10^5$ in PC and $\approx 1.8 \times 10^6$ in PG. The peptide with free cysteines, pSP-C_{L1W}, had coefficients in the same range, $K \approx 4.3 \times 10^5$ and $\approx 2.0 \times 10^6$ in PC and PG, respectively. Although the intrinsic partition coefficients in anionic membranes may be somewhat overestimated due to electrostatic effects that increase the aqueous concentration of peptides at the membrane surface, these results indicate that the peptides have a marked intrinsic affinity for association with lipid membranes. Both peptides have a higher apparent affinity for anionic, as opposed to zwitterionic, bilayers. The calculated partition coefficients represent a free energy of transfer of both peptides from the aqueous to the lipid phase which is around -7.7 and -8.5 kcal/mol for PC and PG membranes, respectively. These values are of a similar order to those found for other membrane-interacting peptides [29,30].

Figure 1 also shows the fluorescence emission spectra of the peptides in the different fractions, obtained in the presence or absence of phospholipids. In the absence of lipids, both peptides have fluorescence spectra with emission maxima at 345–350 nm, typical of tryptophan residues exposed to polar microenvironments. In contrast, the spectra of peptides associated with lipids have maxima shifted to ≈ 330 nm, indicating that association of the peptide with membranes places the tryptophans in a much more hydrophobic environment. The present results indicate that the two peptides show quite comparable behaviour in terms of lipid–protein binding, indicating that thiol blockage by itself does not significantly modify the intrinsic propensity of the sequence to interact with membranes. Although most of the data that follow refer to the properties of the blocked peptide, the free-cysteine version always exhibited qualitatively comparable features.

Lipid–peptide titration

We next analysed the effect of PC or PG lipid/peptide ratio on the fluorescence properties of the peptides. Figure 2 shows the emission fluorescence spectra of pSP-C_{L1Wb} in the absence and in the presence of increasing quantities of PC or PG vesicles.

Both PC and PG membranes produce a progressive increase in peptide fluorescence intensity that was shifted to progressively shorter wavelengths as the lipid concentration increased. The increase in fluorescence emission intensity and the shift of the emission maximum to shorter wavelengths were both markedly larger when the peptide interacted with anionic as opposed to zwitterionic membranes.

Tryptophan quenching by brominated lipids

Figure 3 shows fluorescence emission spectra of pSP-C_{L1Wb} in PC vesicles containing different weight percentages of brominated PC bearing bromines either at the (6,7) or at the (11,12) positions of its acyl chains. Increasing amounts of both brominated lipid probes produce progressive quenching of peptide fluorescence as a result of association of the peptide with the membranes.

Quantitative comparison of quenching by the two brominated lipids allows estimation of the relative position of the peptide tryptophan in the bilayers, provided that the location in the membrane of the two quenching groups is known. This ‘parallax’ method has been used previously by our group to estimate the insertion depth of surfactant protein SP-B in lipid bilayers [26]. Figure 3(B) shows that the extent of quenching of peptide fluorescence by the Br(6,7) isomer is clearly higher than that of

Figure 1 Association of peptides pSP-C_{L1W} and pSP-C_{L1Wb} with egg PC or egg PG vesicles in 50 mM Hepes/150 mM NaCl, pH 7

Lipid/peptide molar ratio was 44. The bar charts show the percentage of peptide and ● the percentage of lipid in each of five ultracentrifuge fractions, separated as indicated by the scheme in the upper right-hand corner. Quantification of the lipid and peptide contents in each fraction was performed as described in the Experimental section. Tryptophan emission fluorescence spectra are also included from the different fractions, 1–5. Spectra were obtained at 25 °C, with an excitation wavelength of 280 nm. Fluorescence emission is presented in arbitrary units (a.u.). F.I., fluorescence intensity.

quenching by the Br(11,12) isomer. Applying the parallax analysis to these data, it was possible to estimate that the peptide tryptophan is located in a region 11 ± 1 Å from the bilayer centre. This value suggests an interfacial disposition in the bilayer for the N-terminal tryptophan of the peptide.

Phospholipid vesicle aggregation

Addition of peptide pSP-C_{L1Wb} to preformed DPPC or DPPG vesicles promotes an almost instantaneous increase in light scattering, at peptide/lipid molar ratios ranging from 1:110 to 1:12

Figure 2 Titration of peptide pSP-C_{L1Wb}-lipid interaction by fluorescence spectroscopy

(A, D) Fluorescence emission spectra of peptide pSP-C_{L1Wb} (4 μg/ml) in the absence (spectrum 1) or presence of 5, 11, 21, 27, 53, 80 or 107 mM (spectra 2–8) egg PC (A) or egg PG (D) vesicles, in 50 mM HEPES buffer, pH 7, containing 150 mM NaCl. The excitation wavelength was 280 nm and the temperature was 25 °C. (B, E) Maximal fluorescence emission intensity and (C, F) wavelength of maximum emission, all plotted versus lipid to peptide molar ratio. Lines in (B), (C), (E) and (F) are visual guides to show the trend in the experimental data.

Figure 3 Quenching of the fluorescence of peptide pSP-C_{L1Wb} by brominated phospholipids

(A) Fluorescence emission spectra at 25 °C of peptide pSP-C_{L1Wb} in PC bilayers containing 0 (●), 10 (○), 20 (■), 30 (□), 40 (▲) and 50% (△; w/w) of Br_(6,7)-PC (left-hand panel) or Br_(11,12)-PC (right-hand panel). The excitation wavelength was 280 nm and the fluorescence intensity is given in arbitrary units. (B) Semilogarithmic plot of the relative fluorescence intensity of the emission spectra of peptide pSP-C_{L1Wb} in PC membranes as a function of the molar fraction of Br-PC in the bilayers.

(Figure 4). This suggests that the peptide causes rapid vesicle aggregation. For any given temperature, the extent of aggregation induced, measured as the increment in absorbance, is greater with anionic vesicles than with DPPC vesicles.

Peptide pSP-C_{L1Wb} induces little aggregation of zwitterionic vesicles at temperatures above their gel-to-liquid crystalline transition temperature (T_m). However, the peptide promotes aggregation of DPPG vesicles at both 25 and 45 °C; temperatures below

Figure 4 Aggregation of phospholipid vesicles induced by peptide pSP-C_{L1Wb}

(A) Aggregation of DPPC (upper panels) or DPPG (lower panels) vesicles induced by the peptide pSP-C_{L1Wb}. Phospholipid vesicles were prepared at 100 $\mu\text{g/ml}$ in 50 mM Hepes/150 mM NaCl, pH 7, buffer which was equilibrated at 25 °C (left) or 45 °C (right). At the time indicated by the arrow, an aliquot of 10 μl of methanol (●) or a concentrated methanolic solution of the peptide was injected to produce final peptide concentrations of 2 (○), 5 (□), 10 (△) and 20 (◇) $\mu\text{g/ml}$, corresponding to lipid/peptide molar ratios of 110, 48, 24 and 12, respectively. Vesicle aggregation induced by the peptide was followed by monitoring the turbidity detected at 400 nm. (B) Percentage of vesicle aggregation induced at 45 °C by the peptide pSP-C_{L1Wb} versus the molar fraction of DPPG in DPPC bilayers, taking aggregation of pure DPPG vesicles as a reference.

and above the T_m of DPPG with sodium as the counterion. Figure 4 also illustrates how the inclusion of a small proportion of PG in zwitterionic membranes is sufficient to make them susceptible to aggregation by the SP-C N-terminal peptide. The presence of as little as 2% DPPG in DPPC bilayers, which are not aggregated by the peptide at temperatures above T_m , confers sensitivity to peptide-induced aggregation. Remarkably, sensitivity to aggregation seems to be maximal already in the presence of 8% PG, i.e. on the order of the physiological content of anionic phospholipid species in surfactant.

Leakage of aqueous content

Perturbation of phospholipid bilayers induced by these peptides also affects membrane permeability. Both pSP-C_{L1W} and pSP-C_{L1Wb} induce leakage of the aqueous content from both zwitterionic and anionic vesicles, above their respective T_m values (Figure 5). Peptide-induced leakage of vesicle contents was always associated with a marked increase in light scattering, implying that leakage is not likely due to a general solubilizing 'detergent-like' effect of the peptide. The time required for the peptide to produce maximum leakage, as well as the maximal amount of fluorescence liberated, were both dependent on peptide concentration.

Differential scanning calorimetry

The effect of pSP-C_{L1Wb} on the thermotropic properties of DPPC bilayers is shown in Figure 6. Increasing amounts of peptide in the bilayers abolish the L_β -P β' transition, which in DPPC occurs at 36.5 °C, and produce progressive broadening and reduction

of the amplitude of the major calorimetric peak associated with the chain-melting transition, without significant shift in the T_m at which this transition occurs (around 41 °C).

Table 1 summarizes the values of the thermodynamic parameters determining the principal thermotropic transition in DPPC bilayers associated with different proportions of peptide. These are derived from the thermograms in Figure 6. The peptide pSP-C_{L1Wb} produces significant effects on both the co-operativity of the transition, characterized by the width $\Delta T_{1/2}$, and the associated enthalpy change (ΔH). These effects suggest the existence of a marked hydrophobic component for the peptide-lipid interaction, the peptide inducing significant perturbations in phospholipid acyl chain packing.

ESR spectroscopy

The effect of pSP-C_{L1Wb} on the gel to liquid-crystalline phase transition of DPPC and DPPG bilayers, as studied by ESR, is illustrated in Figure 7. The outer hyperfine splitting ($2A_{\text{max}}$) of the ESR spectra of DPPC or DPPG bilayers containing, respectively, 1 mol % of a 5-position spin-labelled PC {5-PCSL; 1-acyl-2-[5-(4,4-dimethylloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphocholine} or PG {5-PGSL; 1-acyl-2-[5-(4,4-dimethylloxazolidine-*N*-oxyl)stearoyl]-phosphatidylglycerol}, is plotted against temperature, for both the pure lipid and the lipid-peptide systems containing 40% (w/w) pSP-C_{L1Wb}. The dependence of $2A_{\text{max}}$ on the amount of peptide pSP-C_{L1Wb} in the bilayers was monotonic over a wide range of lipid/protein ratios, up to >40% peptide/lipid by weight, which corresponds to a 1:5.6 peptide/lipid molar ratio (results not shown). Lipid/peptide samples containing 40% (w/w) pSP-C_{L1Wb} were then chosen for

Figure 5 Effect of peptides pSP-C_{L1W}b (left-hand panels) and pSP-C_{L1W} (right-hand panels) on the release of aqueous contents from DPPC or PG vesicles

Peptide concentrations assayed were (from light grey to black) 1, 3, 6 and 12 μ M, corresponding to lipid/peptide molar ratios of 75, 25, 12.5 and 6.25, respectively, and the final lipid concentration was 75 mM. Peptide-induced liberation of the fluorophore ANTS from the vesicles was followed by monitoring fluorescence emission using excitation and emission wavelengths of 353 and 536 nm, respectively. The percentage of leakage induced by any given peptide concentration was estimated taking as a reference the maximum possible leakage, obtained after addition to the samples of an aliquot of Triton X-100 (final concentration 0.5%, v/v). Measurements were made at 45 °C for the samples containing DPPC vesicles and at 25 °C for the samples containing PG vesicles.

most ESR experiments, because at this peptide/lipid ratio the effect of interaction is practically saturated. Dispersions of both DPPC and DPPG alone showed sharp phase transitions at around 40–41 °C, as seen from Figure 7. In DPPC bilayers, the presence of pSP-C_{L1W}b caused a remarkable broadening of the main transition without shifting the temperature of the transition midpoint appreciably. In contrast, the peptide caused a 2 °C shift in T_m of DPPG membranes to lower temperatures. Also, the peptide decreased the amplitude of the change in $2A_{max}$ at the transition, for both DPPC and DPPG bilayers.

The peptide has different effects on the lipid mobility in the gel and the fluid phases of DPPC and DPPG bilayers. In DPPC membranes, pSP-C_{L1W}b increases the outer splittings of the ESR spectra in the lipid fluid phase, apparently without modifying the ESR spectral characteristics of the spin-labelled lipids at temperatures in the gel phase. On the other hand, DPPG–peptide interaction increases $2A_{max}$ of 5-PGSL in both gel and liquid-crystalline phases.

Figure 7 also plots the outer hyperfine splitting of different PC (*n*-PCSL) or PG (*n*-PGSL) spin-label positional isomers versus the position of the spin label group in the *sn*-2 acyl chain of the phospholipid, in the absence and presence of 40% (w/w) pSP-C_{L1W}b at 45 °C. At this temperature, both DPPC and DPPG are in the fluid phase, where the peptide has the strongest

immobilization effects. The bilayers of both phospholipids display the characteristic chain flexibility gradient, both in the absence and in the presence of the peptide. The motional restriction induced by the peptide is always larger at chain positions close to the polar headgroups, where presumably the peptide interacts. At most of the acyl chain segments, the peptide also produces larger immobilizations in DPPG than in DPPC bilayers, as a result of the additional electrostatic component of the peptide/membrane interaction in the former case.

DISCUSSION

Most models that address the structure and disposition of pulmonary surfactant lipopeptide SP-C in phospholipid bilayers and monolayers propose that the N-terminal segment of the protein, which accounts for approximately one-third of its sequence, is strongly associated with the lipids via its palmitoylated cysteines [31]. The other approximately two-thirds of the structure in SP-C consists of a highly hydrophobic α -helix, with a transmembrane orientation in bilayers [13], and which is possibly being directed towards the air spaces when at the air–liquid interface [32]. The results of the present study indicate that the sequence comprised of the 13 N-terminal residues of SP-C has an intrinsic

Figure 6 Differential scanning calorimetry

Shown are differential scanning calorimetry thermograms of DPPC multilamellar suspensions reconstituted in the absence or presence of 4, 10, 20 and 40% by weight of pSP-C_{L1Wb} relative to lipid (lipid/peptide molar ratios of 56, 22.4, 11.2 and 5.6, respectively). Three comparable heating cycles from 20 to 60 °C were scanned for each sample at 30 °C/h.

ability to interact and associate spontaneously with lipid membranes, even in the absence of thioester-linked palmitic chains.

We have previously proposed that the conformation of the N-terminal segment of SP-C could be defined by a high intrinsic propensity to form β -turns [25]. From the sequence of this segment of the protein, the middle part of the turn would be occupied by hydrophobic residues while both ends would contain polar, positively charged amino acids. It is conceivable that such an amphipathic β -hairpin would have intrinsic tendency to associate with the polar/non-polar interface offered by the membrane surface. The β -turn conformation could require partial charge compensation in order to facilitate approach of the two cationic ends of the N-terminal segment (see Figure 8). Negatively charged lipids, such as surfactant PG, could therefore be required to supply appropriate counterions to stabilize the β -hairpin motif. NMR experiments on a 17-residue peptide from the N-terminal

Table 1 Thermodynamic parameters characterizing the effect of peptide pSP-C_{L1Wb} on the main phase transition of multilamellar DPPC suspensions, as determined by DSC

ΔH is the enthalpy of the chain-melting transition, T_m is the temperature of the transition mid-point and $\Delta T_{1/2}$ is the width of the transition.

	ΔH (kcal/mol)	T_m (°C)	$\Delta T_{1/2}$ (°C)
DPPC	8.2	41.8	0.5
DPPC/4 % pSP-C _{L1Wb}	10.2	42.0	0.5
DPPC/10 % pSP-C _{L1Wb}	9.0	42.0	0.5
DPPC/20 % pSP-C _{L1Wb}	6.7	42.0	0.8
DPPC/40 % pSP-C _{L1Wb}	5.3	41.8	1.5

Figure 7 Effect of peptide pSP-C_{L1Wb} on the thermotropic properties and the flexibility profile of DPPC or DPPG bilayers

(A) Effect of temperature on the outer hyperfine splitting constant, $2A_{max}$, in the ESR spectra of DPPC (upper panel) or DPPG (lower panel) bilayers containing 1 mol % of the spin probes 5-PCSL or 5-PGSL, respectively, in the absence (●) and presence (□) of 40% by weight of pSP-C_{L1Wb} relative to lipid. (B) Dependence of $2A_{max}$ on the nitroxide position in *sn*-2 positional spin-labelled PC isomers in DPPC bilayers (above) or *sn*-2 positional PG isomers in DPPG bilayers (below) at 45 °C, in the absence (●) and presence (□) of 40% by weight of pSP-C_{L1Wb}.

sequence of SP-C in zwitterionic dodecylphosphocholine micelles did not detect any defined conformation [11]. Apart from a possible unfavourable geometry of micelles compared with bilayers, we suggest that formation of the β -hairpin motif could be impaired in the absence of anionic lipid species.

Figure 8 represents theoretical membrane-partitioning tendencies in the primary sequence of proSP-C and mature SP-C from several species, according to the scales proposed by Wimley and White [33]. In this figure, the average interfacial hydrophobicity of the different sequence segments are plotted versus residue position. In the same plots, profiles of free energy of transfer from water to octanol and Kyte–Doolittle hydrophobicity are also superimposed. These latter two indices identify highly hydrophobic segments that could potentially partition into the core of lipid bilayers. Two segments in the sequence of SP-C have a free energy that is predicted to be favorable for partitioning into and association with membranes. The C-terminal α -helical segment, including residues 13–35 of the mature sequence, has a high absolute hydrophobicity, as one would expect for the hydrophobic

Figure 8 Model for the disposition of SP-C N-terminal region in phospholipid bilayers

(A) Schematic illustration of the disposition of SP-C and its N-terminal segment in phospholipid bilayers. Polar and non-polar segments in the sequence have been marked as dark and light portions, respectively. Bold lines indicate the surfaces of the bilayers. Negatively charged phospholipids are represented as stabilizers of the amphipathic β -hairpin. (B) Hydrophobicity profiles of proSP-C or mature SP-C sequences from different species. Bold lines are plots calculated using the White–Wimley interfacial hydrophobicity scale [33] based on the free energy of transfer from water to the membrane interface (mean values for a window of six residues). Dashed lines are plots using an scale based on the free energy of transfer from water to octanol. Continuous thin lines are plots using the traditional hydrophobicity index proposed by Kyte and Doolittle [50]. The two segments in the sequence of mature SP-C with highest hydrophobicity are positioned in the ribbon diagram of the protein.

transmembrane region of the protein. The second region in SP-C that has a favourable free energy for interacting with membranes extends from residue 2 or 3 to 8 of the mature sequence and would form the middle part of the N-terminal β -hairpin proposed above.

Our results suggest that the affinity and extent of the interaction of the N-terminal segment of SP-C are considerably greater for anionic than for zwitterionic membranes. Also, the N-terminal segment resides deeper in the interfacial regions of anionic as opposed to zwitterionic membranes (seen, for instance, by a much larger blue-shift of tryptophan fluorescence emission in Figure 2). The importance of an electrostatic component for the lipid–protein interaction of SP-C has been reported previously [15,18,34]. Native SP-C [21], and specifically its N-terminal segment [23], adopts a location and conformation in anionic phospholipids different from that in zwitterionic phospholipids. Positively charged residues in SP-C seem to be essential for the biophysical activity of the protein [17], supporting the idea that the interaction between its N-terminal segment and anionic phospholipid head-groups has a functional significance. Pulmonary surfactant contains about 8% of anionic phospholipids [35], mostly PG and phosphatidylinositol. We propose that these lipids provide sufficient negative charge at surfactant bilayer surfaces to ensure the proper conformation/disposition of the SP-C N-terminal segment, an effect potentially mimicked by the synthetic peptides used in this study.

We propose also that embedding of the N-terminal segment of SP-C in the bilayer surface is probably an important feature in modulating lateral distribution of the protein in surfactant membranes [23].

It has been shown recently that SP-C has the capacity to bind bacterial endotoxin [LPS (lipopolysaccharide)] inserted in membranes [36]. Structural features of the N-terminal segment of SP-C are essential for LPS binding [37]. We suggest that the structure and amphipathicity of the N-terminal segment of SP-C could direct formation of specific SP-C–LPS complexes. The importance of this LPS-binding activity for the anti-bacterial defence mechanisms of the lungs has still to be established.

The results presented in this study further indicate that the N-terminal segment of SP-C not only interacts with lipid membranes but also induces perturbations of the lipid packing that extend more or less deeply into the bilayer. These perturbations cause peptides from this region of SP-C to induce lipid vesicle aggregation and leakage of their aqueous contents. At temperatures below T_m , the peptides are probably excluded from the ordered lipid phases and do not cause substantial effects on the packing of the phospholipid acyl chains. Peptide-induced aggregation of phospholipid vesicles at those temperatures could be induced by insertion of the peptide into dislocations at the gel–fluid boundaries, but also by electrostatic lipid–protein

interactions at the surface of anionic membranes. Above T_m , in the absence of such dislocations, the peptide only aggregates anionic membranes, probably requiring an electrostatic component, which is independent of the physical state of the membranes themselves. Lipid-peptide interactions producing leakage at temperatures above T_m may require a different location or disposition of the peptide in the membranes than that at temperatures below T_m . A preliminary analysis of the kinetics of peptide-induced leakage and its dependence on lipid/peptide ratio [38] seems to indicate that leakage could proceed via formation of 'pore-like' structures. Formation of peptide transmembrane pores would necessarily imply peptide oligomerization occurring at the surface of the membranes, followed by insertion of the peptide assemblies in a transmembrane orientation. A similar behaviour has been proposed for other amphipathic peptides that were able to aggregate and form pores depending on the lipid membrane phase [38]. Interestingly, membranes formed from pulmonary surfactant extracts containing the hydrophobic proteins SP-B and SP-C, have been reported to contain discrete pores or channels [39], raising the question of a possible physiological significance for protein-promoted channel-like structures in surfactant membranes.

The membrane-perturbing properties of the N-terminal segment might well underly the ability of SP-C to induce mixing of lipid components between vesicles [14], or to catalyse bilayer-to-monolayer phospholipid transfer [40]. Both differential scanning calorimetry and especially spin-label ESR experiments, allow us to conclude that the SP-C N-terminal region has significant effects on the order/mobility of segments of the phospholipid acyl chains located deep in the hydrophobic core of the membranes. The hydrophobic component of the peptide-lipid interaction is present even in anionic membranes, where electrostatic interactions contribute strongly. The dominant hydrophobic component of the SP-C-phospholipid interaction has been widely studied [16,41,42], always being attributed to lipid-protein interactions arising mainly from the transmembrane hydrophobic α -helix, which extends over residues 13–35 of the mature protein. The present results provide evidence that interaction of the N-terminal segment with membranes also affects the hydrophobic interior of the membrane, even in the absence of cysteine thio-acylation. This interaction might be essential for certain roles of SP-C in the lung such as promoting the bilayer-monolayer transitions that bring the surface-active lipid molecules to the alveolar air-liquid interface [43]. Palmitoylation probably has a rather subtle role in modulating conformation and lipid-protein interactions of the N-terminal tail in native SP-C.

Several studies published in recent years have suggested that the interfacial surface-active monolayer is associated *in vivo* with bilayer-based structures near and below the surface. These bilayers could act as a reservoir to supply new surfactant molecules to the interface during successive respiratory cycles [20,44]. The lipopeptide SP-C has been proposed to participate in the establishment and maintenance of such interfacial reservoirs, with the N-terminal segment of the protein assuming a principal role [45–47]. It has been shown recently that acylation of this region could be essential to maintain association of the reservoirs with compressed states of the interfacial film at the end of expiration [46,48]. Upon inspiration, the perturbations introduced by the N-terminal protein segment in surfactant bilayers and monolayers could catalyse the formation of the intermediate structures necessary to promote a rapid and continuous transfer of surfactant molecules to the interface [49].

The N-terminal region of pulmonary surfactant protein SP-C has an intrinsically dynamic behaviour in terms of lipid-protein interactions and membrane perturbations. The physico-chemical properties of this segment of the protein necessarily have to be

considered to understand the role of SP-C in surfactant biophysics at the alveolar spaces, and when designing SP-C-based surfactant preparations for respiratory therapies.

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