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Effect of acylation on the interaction of the N-Terminal segment of pulmonary surfactant protein SP-C with phospholipid membranes

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ABSTRACT

SP-C, the smallest pulmonary surfactant protein, is required for the formation and stability of surface-active films at the air–liquid interface in the lung. The protein consists of a hydrophobic transmembrane α-helix and a cationic N-terminal segment containing palmitoylated cysteines. Recent evidence suggests that the N-terminal segment is of critical importance for SP-C function. In the present work, the role of palmitoylation in modulating the lipid–protein interactions of the N-terminal segment of SP-C has been studied by analyzing the effect of palmitoylated and non-palmitoylated synthetic peptides designed to mimic the N-terminal segment on the dynamic properties of phospholipid bilayers, recorded by spin-label electron spin resonance (ESR) spectroscopy. Both palmitoylated and non-palmitoylated peptides decrease the mobility of phosphatidylcholine (5-PCSL) and phosphatidylglycerol (5-PGSL) spin probes in dipalmitoylphosphatidylcholine (DPPC) or dipalmitoylphosphatidylglycerol (DPPG) bilayers. In zwitterionic DPPC membranes, both peptides have a greater effect at temperatures below than above the main gel-to-liquid-crystalline phase transition, the palmitoylated peptide inducing greater immobilisation of the lipid than does the non-palmitoylated form. In anionic DPPG membranes, both palmitoylated and non-palmitoylated peptides have similar immobilizing effects, probably dominated by electrostatic interactions. Both palmitoylated and non-palmitoylated peptides have effects comparable to whole native SP-C, as regards improving the gel phase solubility of phospholipid spin probes and increasing the polarity of the bilayer surface monitored by pK shifts of fatty acid spin probes. This indicates that a significant part of the perturbing properties of SP-C in phospholipid bilayers is mediated by interactions of the N-terminal segment. The effect of SP-C N-terminal peptides on the chain flexibility gradient of DPPC and DPPG bilayers is consistent with the existence of a peptide-promoted interdigitated phase at temperatures below the main gel-to-liquid-crystalline phase transition. The palmitoylated peptide, but not the non-palmitoylated version, is able to stably segregate interdigitated and non-interdigitated populations of phospholipids in DPPC bilayers. This feature suggests that the palmitoylated N-terminal segment stabilizes ordered domains such as those containing interdigitated lipids. We propose that palmitoylation may be important to promote and facilitate association of SP-C and SP-C-containing membranes with ordered lipid structures such as those potentially existing in highly compressed states of the interfacial surfactant film.

1. Introduction

S-acylation of cysteine residues in proteins is decisive to function, e.g., by modulating membrane association and dynamics, and by influencing protein stability. In several cases, acylation is necessary for complete association of the protein, or its domains, with the membrane, and it additionally modulates lipid–protein interactions [1]. Palmitoylation of proteins with only one transmembrane domain usually occurs juxtaposed with the transmembrane region, as is the case with the pulmonary surfactant protein SP-C [2].

SP-C is not strictly required to initiate respiratory function at birth, as shown by survival of SP-C knock-out mice [3]. However, the lungs of these SP-C-deficient animals appear to be intrinsically unstable. Consistent with this, genetic deficiencies in the structure and expression of the SP-C gene have been found in patients developing chronic respiratory diseases [4]. *In vitro*, SP-C has been shown to promote interfacial phospholipid adsorption and to stabilize interfacial monolayers that are subjected to dynamic compression–expansion cycling [5,6]. SP-C seems also to participate in generating the surface-attached reservoir of surfactant that provides a continuous supply of surface-active molecules to replenish the interfacial film during successive

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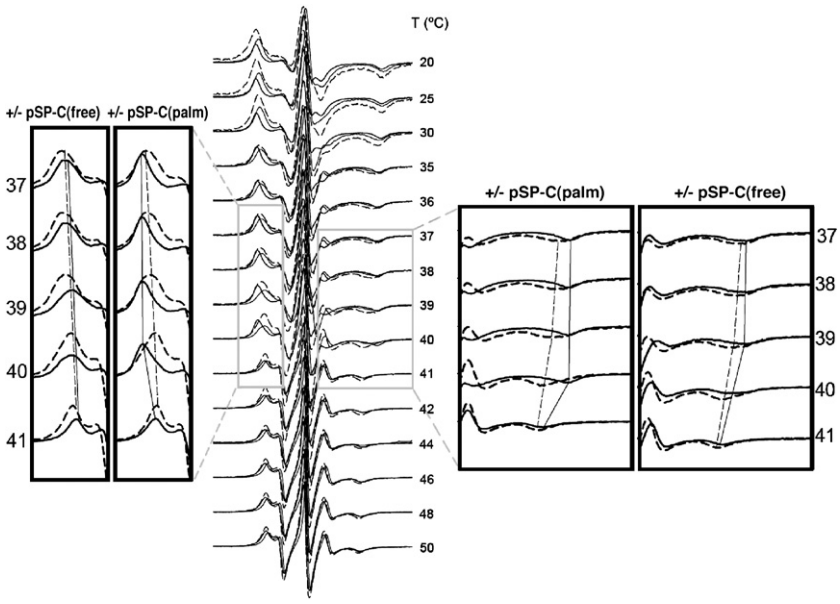

Fig. 1. ESR spectra of a 5-PCSL spin-label in dipalmitoylphosphatidylcholine (DPPC) bilayers, in the presence or in the absence (dashed line) of 40 wt% of the acylated peptide pSP-C $(Palm)_2$ (solid black line) or the non-palmitoylated peptide pSP-C(free) (solid grey line), at the temperatures indicated. Lines connecting the different spectra are visual guides to illustrate temperature-dependent reduction in the total spectral width. The total scan range was 100 G. Inserts on the left- and right-hand sides show expanded details of the low- and high-field peaks of the ESR spectra in the absence (dashed line) or presence (solid line) of the palmitoylated or non-palmitoylated peptide, as indicated.

respiratory cycles [7]. All SP-C sequences identified so far possess one or two cysteines at the N-terminal region, including the very recently reported SP-C sequence from the toad *Xenopus* [8–10], and in all cases investigated these N-terminal cysteines are stoichiometrically palmitoylated. Numerous biophysical studies of SP-C have indicated that palmitoylation of the N-terminal region increases hydrophobicity, which is important for stabilization of the α-helical structure [11,12], favoring the interaction of the protein with surfactant phospholipids. Moreover, palmitoylation plays an important role in the correct adsorption and reincorporation of surfactant material into the surface-active film [13,14], and in the stability of these films when subjected to repetitive compression–expansion cycling [15]. In contrast, other studies did not find significant differences when comparing the behaviour of acylated and deacylated forms of SP-C in interfacial films [16,17]. On the other hand, non-palmitoylated recombinant forms of SP-C produced in bacteria show similar, if not better, ability to promote formation of phospholipid-based interfacial films than does the native palmitoylated protein purified from mammalian lungs [18]. In the same direction, surfactant preparations containing non-palmitoylated recombinant SP-C forms as the only protein component have shown efficiency in restoring pulmonary function *in vivo* [19–21].

In recent years, studies with synthetic peptides mimicking the sequence of the N-terminal segment of SP-C have shown that this region of the protein possesses intrinsic capability to interact with and to perturb phospholipid bilayers [22] and monolayers [23], even in the absence of palmitoylation. The question is, therefore, how palmitoylation modulates the conformation and/or the manner in which the N-terminal segment interacts with phospholipids. Palmitoylation of the SP-C N-terminal segment, for instance, seems to be important in maintaining the association of this protein segment with highly compressed interfacial films [24,25], such as those formed at the end of exhalation during respiration. Whether association with compressed states requires a particular conformation or disposition of the palmitoylated segment in the lipid layers, or is maintained exclusively through the interfacial insertion of the protein acyl chains, is not clear. A major objective of the present work is to obtain further insight into the effect of palmitoylation on the mode and extent of lipid–protein interactions established by the N-terminal segment of SP-C in phospholipid bilayers, by using spin-label electron spin resonance (ESR) spectroscopy.

2. Materials and methods

2.1. Materials

Chloroform (Chl) and methanol (MeOH) were HPLC-grade solvents from Scharlau (Barcelona, Spain). The lipids, dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG), were purchased from Avanti Polar Lipids (Birmingham, AL, USA). Spin-labelled phosphatidylcholine and phosphatidylglycerol with the nitroxide group at different positions in the *sn*-2 acyl chain were synthesized as described by Marsh and Watts [26]. The spin-labels were stored at −20 °C in Chl/MeOH (2/1, v/v) solutions at a concentration of 1 mg/ml.

2.2. Peptide synthesis and purification

13-residue peptides were synthesized by Fmoc chemistry, as described elsewhere [24,27], taking the sequence of porcine SP-C as a template. The peptide named pSP-C (free) (NH_2-L-R-I-P-C-C-P-V-N-L-K-R-L-$CONH_2$), has the two cysteines of the native sequence in free sulfhydryl form. The other peptide, named pSP-C$(Palm)_2$ (NH_2-L-R-I-P-C_{Palm}-C_{Palm}-P-V-N-L-K-R-L-$CONH_2$) is palmitoylated at Cys5 and Cys6, as in the N-terminal segment of native SP-C. Both synthetic products were purified to >95% (HPLC) homogeneity by semipreparative HPLC on C8-silica linear acetonitrile–water gradients [24,27]. The purified materials had the correct amino acid analysis and gave the expected molecular mass by MALDI-TOF mass spectrometry.

2.3. Lipid/peptide samples

As a membrane model, lipid and lipid/peptide bilayers were prepared using either DPPC or DPPG as a host phospholipid matrix. These species have been widely used as models of pulmonary surfactant. DPPC is the main phospholipid species in surfactant and also that providing the main surface activity [6,28]. Phosphatidylglycerol (PG) is the main anionic phospholipid in surfactant, bearing also a fair proportion of saturated acyl chains. Although pulmonary surfactant also contains a substantial proportion of unsaturated phospholipids, it has been found that saturated PC and PG species segregate in surfactant membranes and films [29–31]. Pure DPPC or DPPG bilayers show well-defined thermotropic behaviour, including sharp temperature-driven gel-to-fluid phase transitions, which allow direct determination of the effect of proteins and peptides on both gel and fluid membrane phases. For ESR experiments, appropriate amounts of DPPC or DPPG and the selected spin-labelled lipid (1 mol%) were dissolved in Chl/MeOH (2/1, v/v), and mixed with the desired amount of peptide. Organic solvent was then removed, first under a N_2 stream, and later by placing the samples under vacuum overnight. Multilamellar suspensions were prepared by hydrating the samples in 100 μl of buffer (50 mM HEPES, 150 mM NaCl, pH 7), and the reconstituted material

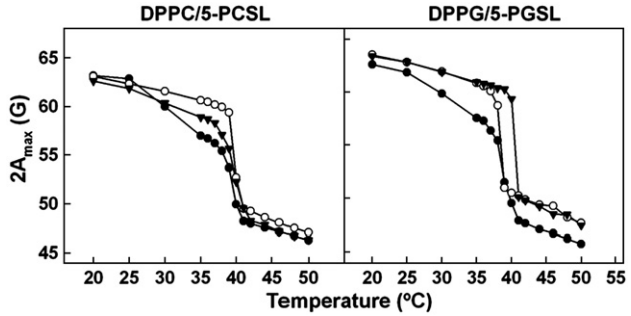

Fig. 2. Temperature dependence of the outer splitting, $2A_{max}$, of the ESR spectra of a 5-PCSL probe in DPPC membranes (left panel) or a 5-PGSL probe in DPPG membranes (right panel), in the absence (●) or in the presence of 40 wt% of the acylated peptide pSP-C(Palm)$_2$ (○), or the non-acylated peptide pSP-C(free) (▼).

was pelleted in the 100-μl capillary tubes used for ESR spectroscopy by centrifugation at 3000 rpm in a bench centrifuge. In some experiments, the lipid and lipid/protein complexes were suspended in buffer at different pH values.

2.4. 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, and 100 G scan range. Several scans, typically 3–5, were accumulated to improve the signal-to-noise ratio. See Perez-Gil et al. (1995) [32] for details of spectral analysis. Figures represent illustrative data after repeating the experiments with three independently-synthesized batches of peptides, which showed similar qualitative behavior and comparable quantitative hyperfine splittings.

3. Results

3.1. Temperature dependence

Fig. 1 shows the temperature dependence of the ESR spectra of 5-PCSL spin-labelled phosphatidylcholine in membranes of dipalmitoylphosphatidylcholine (DPPC) in the presence and absence of 40 wt% of the non-palmitoylated, pSP-C(free), or the dipalmitoylated N-terminal SP-C peptide, pSP-C(Palm)$_2$. For comparison, details of the low-field and high-field spectral lines of 5-PCSL, in the presence and absence of the two peptide sequences, have also been included in the figure. All the spectra show, in the absence (dashed lines) and in the presence (solid lines) of peptide, a pronounced temperature dependence that is characterized by an abrupt narrowing of the spectral extent at the chain-melting transition, around 41 °C. Differences are detected between the spectra from membranes with and without either peptide, both in the gel and fluid bilayer phases. The left panel of Fig. 2 illustrates the temperature dependence of the outer hyperfine splitting, $2A_{max}$, from the spectra of 5-PCSL in DPPC membranes in the presence of the palmitoylated peptide pSP-C(Palm)$_2$, or of the non-acylated peptide pSP-C(free), both at 40 wt%, relative to DPPC alone. The sharp chain-melting transition at 40–41 °C is preserved in the presence of 40 wt% peptide. Increases in $2A_{max}$ are induced by both peptides in the gel and fluid phases, however, and the largest difference is found in the intermediate gel phase, above the pretransition at ca. 30 °C. The palmitoylated peptide essentially abolishes the DPPC pretransition, removing the cooperative decrease in $2A_{max}$ that takes place on transition from the L_{β}' gel phase to the intermediate P_{β}' phase of DPPC bilayers. The effect of the non-acylated peptide on the shallow regions of the membranes sensed by the 5-PCSL probe, at temperatures above the pretransition, is in the same direction as that induced by the palmitoylated peptide, but is considerably weaker.

Somewhat similar results are found on the interaction of both peptides with DPPG membranes, except that the effect of the non-acylated peptide is relatively greater. The right-hand panel of Fig. 2 gives the temperature dependence of the outer hyperfine splitting of a 5-PGSL spin-label in dipalmitoyl phosphatidylglycerol membranes, with or without the palmitoylated or non-acylated peptide. In the presence of either peptide, the pretransition at about 30 °C is practically completely abolished and a very sharp transition between 40 and 41 °C can be observed. This effect can be explained by the stronger electrostatic interaction of the cationic peptide with the anionic headgroups of the DPPG membrane. Inclusion of the non-acylated peptide in DPPG membranes shifts the transition to temperatures 2–3 °C higher, corresponding to an electrostatic neutralisation of the surface charge [33].

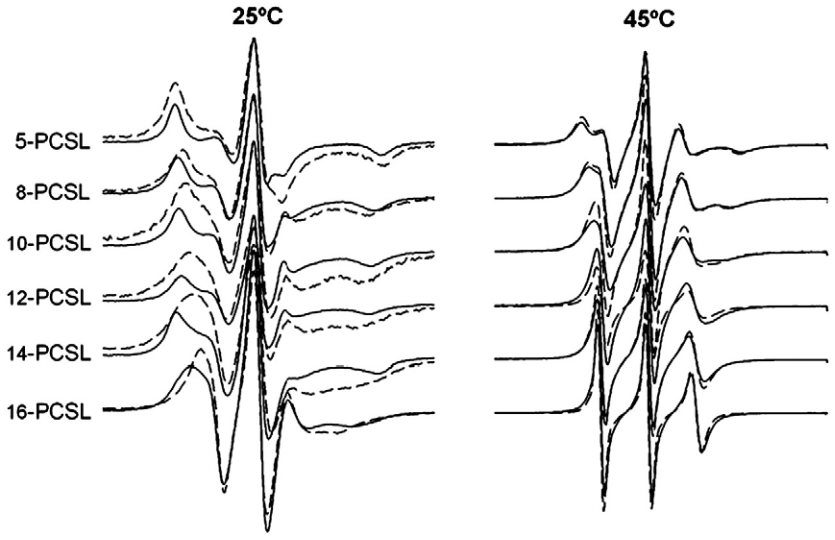

Fig. 3. ESR spectra of different *n*-PCSL spin-label isomers in DPPC membranes, in the presence (solid line) or in the absence (dashed line) of 40 wt% of the acylated peptide pSP-C(Palm)$_2$, at 25 °C in the gel phase (left) or at 45 °C in the fluid phase (right). Total scan range of the spectra, 100 G.

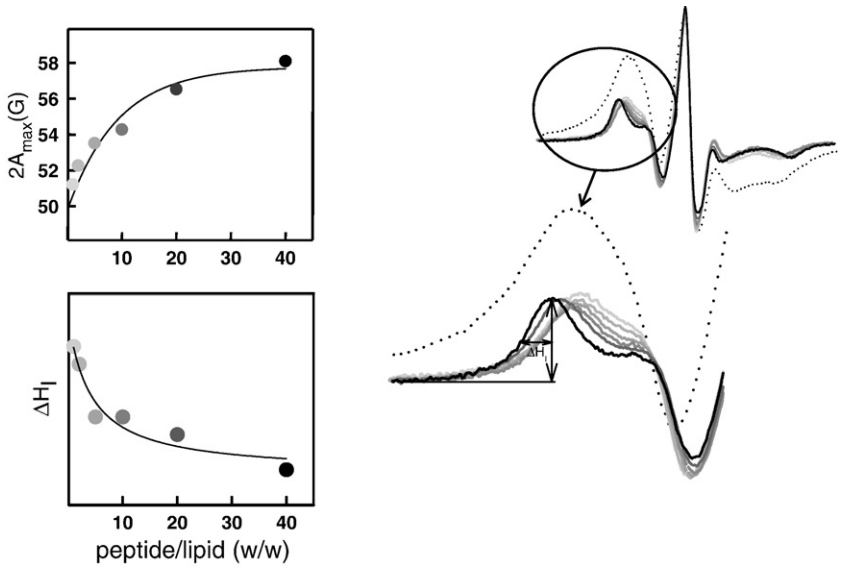

Fig. 4. Right: ESR spectra of a 12-PCSL probe in gel-phase DPPC bilayers at 25 °C, in the absence (dotted line) or in the presence of increasing proportions (light grey to black lines indicate 2, 5, 10, 20 or 40 wt%) of peptide pSP-C(Palm)$_2$. Left panels: outer hyperfine splitting, $2A_{max}$ (upper), and low-field line width at half-height, ΔH_l (lower), as a function of peptide/lipid ratio in the membranes.

3.2. Positional dependence

Fig. 3 shows the ESR spectra of *n*-PCSL phosphatidylcholines spin-labelled at different positions, *n*, of the *sn*-2 chain in DPPC membranes, with and without dipalmitoylated peptide. The left-hand panel refers to membranes in the gel phase at 25 °C and the right-hand panel to membranes in the fluid phase at 45 °C. A uniformly small perturbation in mobility by the peptide is seen throughout the fluid phase. In the gel phase, however, the perturbation by the acylated peptide is greatest towards the terminal methyl end of the chain, where the mobility in a non-interdigitated DPPC gel phase is appreciable (see e.g. [34]). Additionally, for label positions in the middle of the chain, *n*=8–12, the spin–spin broadening that is evident in the gel phase of the DPPC alone [35] is removed in the presence of 40 wt% of the palmitoylated peptide. This effect on the chain packing of gel-phase lipids is a feature that the N-terminal peptide shares in common with native SP-C [32]. Fig. 4 (right) shows that exclusion of the 12-PCSL spin probe from the gel phase of DPPC at 25 °C is substantially reduced already by the addition of 1 wt% of pSP-C(Palm)$_2$. The dependence of the effective hyperfine splitting, $2A_{max}$, and low-field linewidth, ΔH_l, on peptide content (see Fig. 4, left) demonstrates that the peptide–lipid interaction is close to saturation at 40 wt% pSP-C(Palm)$_2$. The non-palmitoylated sequence

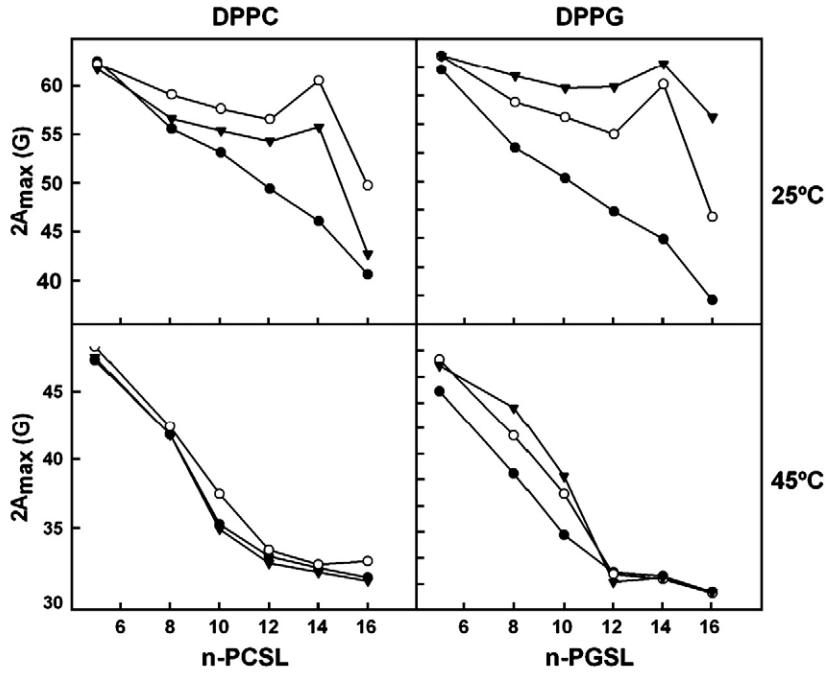

Fig. 5. Positional (*n*) dependence of the outer splitting, $2A_{max}$, in the ESR spectra of *n*-PCSL spin-label isomers in DPPC membranes (left side), and of *n*-PGSL spin-label isomers in DPPG membranes (right side), in the absence (●) or in the presence of 40 wt% palmitoylated peptide pSP-C(Palm)$_2$ (○) or the non-acylated peptide pSP-C(free) (▼), at 25 °C (gel phase; upper panels) or 45 °C (fluid phase; lower panels).

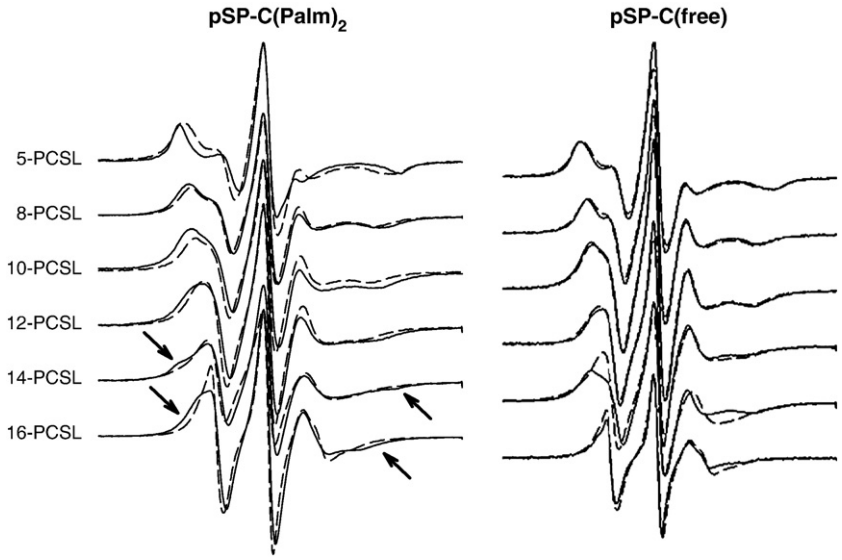

Fig. 6. ESR spectra of different *n*-PCSL spin-label isomers in DPPC membranes at 37 °C, in the absence (dotted lines) or in the presence (solid lines) of 40 wt% of the palmitoylated peptide pSP-C(Palm)$_2$ (left column) or the non-acylated peptide pSP-C(free) (right column). Total scan width: 100 G. Arrows indicate the position of the immobilized spectral component that is attributed to a lipid population in an interdigitated (L^i_β) phase.

had similar qualitative effects in terms of improving the solubility of the spin probe in the gel phase (not shown).

Fig. 5 (lower left-hand panel) shows the dependence of the *n*-PCSL outer hyperfine splitting, $2A_{max}$, on position, *n*, of chain spin-labelling for fluid DPPC bilayers, in the presence and absence of the dipalmitoylated or non-acylated peptide. The chain flexibility profile with increasing *n*, which is characteristic of spin-labelled lipid chains in fluid phase membranes, is preserved in the presence of both peptides. Only modest increases in $2A_{max}$ are found at any chain position in the fluid phase. In the gel phase at 25 °C, both peptides perturb the mobility profile, in the sense that the values of A_{max} for $n=14$ become more comparable to those for $n=5$, in the presence of peptide (see Fig. 5, upper left). This removal of the mobility gradient is a feature associated with the formation of an interdigitated gel phase, L^i_β, in DPPC bilayers (see e.g. [34,36]). The right-hand panels of Fig. 5 give the positional dependences of the outer hyperfine splittings of the *n*-PGSL spin-labels in gel and fluid DPPG membranes, with and without the palmitoylated or non-acylated peptides. Overall, much the same differential features are exhibited in the interaction of the two peptides as with membranes of DPPC, except that the perturbations of DPPG membranes by the peptides are considerably larger, particularly in the fluid phase. As already mentioned above, it is possible that in the absence of palmitoylation the electrostatic interactions between the cationic peptide and the anionic phospholipid headgroups dominate and cause the peptide to interact differently with anionic bilayers, as compared with zwitterionic bilayers.

3.3. Phase coexistence

Fig. 6 shows the ESR spectra of different *n*-PCSL spin-labelled positional isomers in DPPC membranes at 37 °C, i.e., corresponding to the intermediate P'_β phase in the absence of peptide. A notable feature is the coexistence of two spectral components for spin-labels situated towards the terminal methyl position (i.e., at $n=14$–16), in the presence of the palmitoylated peptide, pSP-C(Palm)$_2$. This suggests that, at 37 °C, the membranes containing the dipalmitoylated peptide are in a state of coexistence between interdigitated and non-interdigitated gel phases, because the outer spectral component that is characteristic of the L^i_β-phase is more strongly immobilised ($2A_{max}$ ~60 G for 14-PCSL, see Table 1) than in the peptide-free P'_β-phase

Table 1
Outer hyperfine splittings, in the presence and absence of peptide pSP-C(Palm)$_2$ or pSP-C(free), of phosphatidylcholine spin-labelled at the 14th or 16th position of the *sn*-2 acyl chain, incorporated in DPPC bilayers at 37 °C

$2A_{max}$ (gauss)			
	DPPC	DPPC/pSP-C(free)	DPPC/pSP-C(palm)
14-PCSL	33.8	48.3	59.6, 34.2
16-PCSL	32.3	32.7	43.3, 32.5

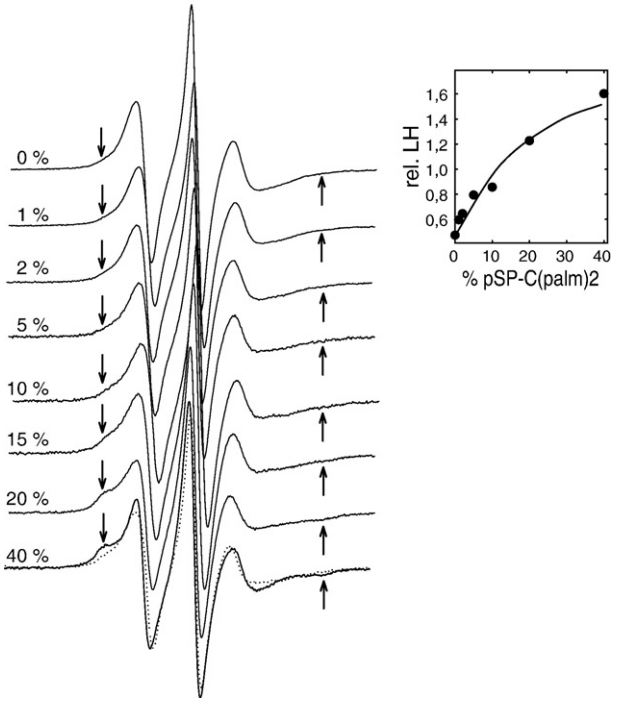

Fig. 7. ESR spectra of a 14-PCSL spin probe in DPPC membranes at 37 °C in the presence of increasing amounts (wt%) of the palmitoylated peptide pSP-C(Palm)$_2$. For comparison, the ESR spectrum of membranes containing 40 wt% of the non-palmitoylated peptide is also included (dotted line). Total scan width: 100 G. Arrows indicate the position of a second, more immobilized, spectral component. Inset: ratio of low-field lineheights of the immobilized relative to the non-immobilized component, with the latter normalized with respect to the peak-to-peak linewidth squared, versus the peptide to lipid ratio.

Table 2
Differences in the outer hyperfine splittings, in the presence and absence of peptide pSP-C(Palm)2 or pSP-C(free), of different phospholipid species spin-labelled at the fifth position of the sn-2 acyl chain, incorporated in DPPC bilayers at 45 °C

	$2\Delta A_{max}$ (gauss)		$2\Delta\Delta A_{max}$ [a] (gauss)		
	pSP-C(free)	pSP-C(palm)	pSP-C(free)	pSP-C(palm)	SP-C [b]
5-PCSL	0.2	1	0	0	0
5-PESL	0.1	0.8	−0.1	−0.2	−0.4
5-PGSL	0.1	0.7	−0.1	−0.25	−0.4
5-SMSL	0.3	0.7	0.1	−0.3	
5-PASL pH 8.5	0.9	0.7	0.8	−0.3	
5-PSSL	0	0.1	−0.2	−0.8	−0.5
5-SASL pH 8.5	0	0	−0.2	−1	−1.4

[a] $2\Delta\Delta A_{max} = 2\Delta A_{max} - 2\Delta A_{max}$ (5-PCSL).
[b] Native, full-length SP-C. Data from ref. [32].

($2A_{max}$ ~34 G for 14-PCSL). The coexistence of two spectral components is indicated at other positions of spin-labelling in the left-hand part of Fig. 6. It is manifested by a shoulder on the low-field peak in the spectra of 8-, 10- and 12-PCSL. Also, two components are clearly resolved in the spectra of 5-PCSL in the pretransition region at 38–40 °C for DPPC samples containing 40 wt% palmitoylated peptide (solid lines in Fig. 1). Note that, although the spectral anisotropy of 14-PCSL is increased by the non-palmitoylated peptide, pSP-C(free), the outer hyperfine splitting ($2A_{max}$ ~50 G) still remains considerably smaller than that for an interdigitated gel phase.

Fig. 7 shows the dependence of the two-component spectra of 14-PCSL at 37 °C on the content of palmitoylated peptide in DPPC membranes. The relative intensity of the outer, motionally restricted, spectral component increases progressively with increasing peptide content, while its lineshape remains unchanged beyond 10 wt% with $2A_{max}$ ~60 G. This suggests strongly that at 37 °C the palmitoylated peptide is preferentially associated with domains of interdigitated lipid, the formation of which is driven by the lipid–peptide interaction. As the content of palmitoylated peptide in the membrane increases, either the size or number of the interdigitated domains increases. This is a property that is associated predominantly with the palmitoylated peptide. The spectrum of 14-PCSL in the presence of the non-acylated peptide shows much less contribution of immobilized components, which cannot be clearly resolved. The palmitoyl chains attached to the peptide therefore must potentiate formation of an interdigitated gel phase. The insert in Fig. 7 shows how the proportion of the more immobilized component titrates with increasing peptide/lipid ratio, as followed by the spectral lineheight of the immobilized (interdigitated-like) component, relative to that of the less immobilized (non-interdigitated) component.

3.4. Phospholipid selectivity

To evaluate potential selectivity of the N-terminal segment of SP-C in the interaction with different phospholipid classes, the ESR spectra of different phospholipid species spin-labelled at the fifth position of the *sn*-2 chain were recorded in DPPC bilayers, in the presence or absence of peptide [37]. Spin probes tested include phosphatidylcholine (5-PCSL), phosphatidylethanolamine (5-PESL), phosphatidylglycerol (5-PGSL), sphingomyelin (5-SMSL), phosphatidic acid (5-SASL), phosphatidylserine (5-PSSL) and stearic acid (5-SASL). Spectra of 5-PASL and 5-SASL were obtained at pH 8.5, to avoid the overlapping of components arising from protonated and deprotonated species which coexist at lower pH values. Either peptide was added at 40 wt% and measurements were made at a fixed temperature of 45 °C in the fluid phase, to ensure miscibility of host lipids and probes.

Table 2 gives the increase in the outer hyperfine splitting, $2\Delta A_{max}$, of each spin probe due to the presence of the corresponding peptide, with respect to the same probe in pure lipid bilayers. $2\Delta\Delta A_{max}$ is the difference between $2\Delta A_{max}$ calculated for each lipid probe and that obtained using the 5-PCSL phosphatidylcholine spin-label. For comparison, data on $2\Delta\Delta A_{max}$ obtained previously with full-length native porcine SP-C [32] are also included in the Table. In general terms, the palmitoylated peptide produced higher values of $2\Delta A_{max}$ than those caused by the non-acylated peptide, confirming the greater ability of the palmitoylated sequence to perturb and immobilize phospholipid acyl chains in bilayers. Interestingly, pSP-C(free) shows very small values of $2\Delta A_{max}$ for all spin-labelled lipids except for the interaction with the dianionic phospholipid 5-PASL, pointing again to a greater electrostatic effect with the non-acylated peptide. The absolute values of $2\Delta A_{max}$ produced by the full-length SP-C molecule [32] are much larger than those produced by the N-terminal palmitoylated segment, indicating that most of the immobilizing effect of the wild-type protein is mediated by the hydrophobic transmembrane segment. However, the selectivities relative to phosphatidylcholine, which are given by the values of $2\Delta\Delta A_{max}$ in Table 2, are comparable for full-length protein and palmitoylated peptide. This suggests that the limited lipid selectivity that is exhibited by SP-C resides in the palmitoylated N-terminal segment.

3.5. Effect on pKs at the membrane surface

In order to determine the influence of the SP-C N-terminal peptide on the polarity of the membrane surface, we studied the effect of acylated and non-acylated peptides on the ionization properties of a stearic-acid spin probe. This was detected by pH-dependent spectral changes related to differences in mobility of the protonated and deprotonated species under different pH conditions (see [38]). A shift in pK_a of the stearic acid carboxyl group in the presence of the peptide could arise from a change in polarity in the region of the lipid headgroups, an electrostatic contribution of the peptide charge, and/or the energetics of lipid–protein interaction [38,39]. Fig. 8 shows the spectra of a 5-SASL stearic-acid spin probe in DPPC bilayers at 45 °C, at different pH values. At pH 5.5, the reduced hyperfine anisotropy indicates the relatively high mobility of the fully protonated stearic acid in fluid DPPC bilayers. On the other hand, at pH 8.5, the spectral anisotropy increases because of the higher orientational ordering of the anionic form of the fatty acid, which is more firmly anchored at the membrane surface [40,41]. At pH 7, in the absence of peptides, both charged and uncharged forms of stearic acid coexist, as seen from the two overlapping spectral components. This indicates that pH 7 is close

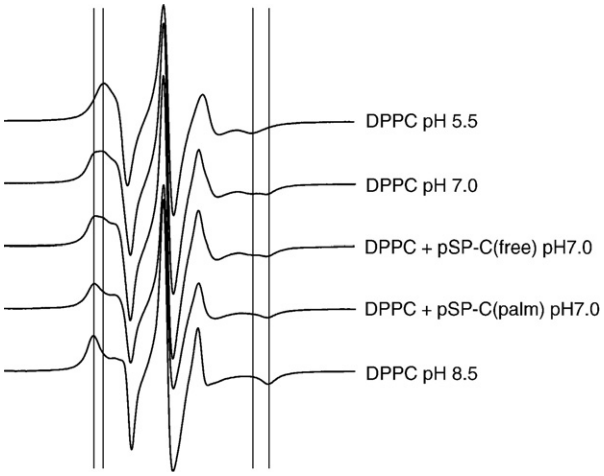

Fig. 8. ESR spectra of 5-SASL, a free fatty acid spin probe, in DPPC membranes at the pH values indicated, at 45 °C, in the absence or in the presence of the palmitoylated peptide pSP-C(Palm)$_2$ or the non-acylated peptide pSP-C(free). Total scan width: 100 G. The positions of the outer hyperfine peaks in the low- and high-field regions of the spectra are indicated by vertical lines.

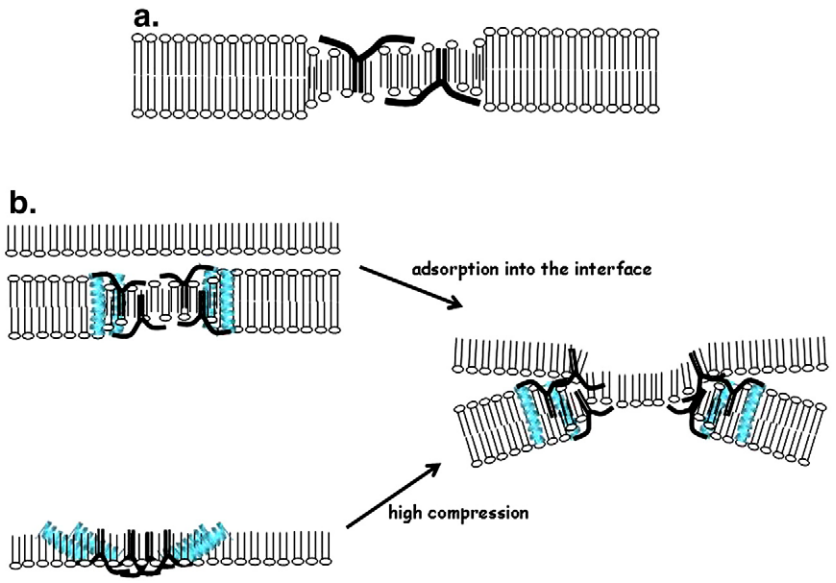

Fig. 9. a) Cartoon illustrating the configuration of a peptide-promoted interdigitated structure in phospholipid bilayers. b) Model for the possible participation of an SP-C-promoted interdigitated intermediate in facilitating transfer of phospholipids to the air–liquid interface, and in the compression-driven formation of surface-associated surfactant structures.

to the ionization pK_a of stearic acid at the DPPC bilayer surface, as reported previously [32]. The spectra of the stearic acid probe in the presence of the peptides show that the palmitoylated peptide, but not the non-palmitoylated peptide, shifts the ionization equilibrium of the fatty acid to lower pH. In the presence of pSP-C(Palm)$_2$, 5-SASL gives a spectrum at pH 7, which has an outer hyperfine splitting comparable to that obtained at pH 8.5 in the absence of peptide. Hence the fatty acid is fully deprotonated at pH 7 in the presence of pSP-C(Palm)$_2$, whereas the protonation state at pH 7 remains essentially unchanged in the presence of the non-acylated pSP-C(free) peptide. Interaction with the palmitoylated N-terminal segment, but not with the non-palmitoylated peptide, therefore increases the polarity of the membrane surface, which gives rise to a decrease in interfacial pK_a of stearic acid. (Note that the pK_a of stearic acid in aqueous solution is about 5.0). In this sense, the palmitoylated N-terminal segment of SP-C is mimicking the effect of the whole protein, which is also able to increase the apparent polarity of the bilayer surface, as monitored by the pH titration of SASL probes [32]. Such an effect cannot be attributed purely to the accumulation of surface charge, because the non-palmitoylated SP-C peptide possesses an identical cationic charge but does not produce equivalent pK shifts. The different effect that pSP-C(palm)$_2$ exerts compared with pSP-C(free) underlines the role of acylation in modulating the way that the N-terminal segment of SP-C interacts with the surface of phospholipid layers.

4. Discussion

The data provided by the present study show how palmitoylation of two adjacent cysteine residues in the N-terminal segment of SP-C affects its interaction with phospholipid bilayers. It has been reported that the presence of palmitic chains can be important for modulating the conformation of the N-terminal segment, and may also influence the structure of the remainder of the protein. Absence of the acyl chains has been related directly to transformation of the α-helical structure of SP-C into β-sheet conformers, leading to formation of SP-C aggregates and amyloid-like fibril structures [11,12]. Both IR spectroscopy [24] and CD spectroscopy (data not shown) show a higher proportion of α-helical structure in the palmitoylated peptide than in the non-palmitoylated peptide. Hence, differences in the conformational state of this region could be responsible in part for differences in lipid–protein and/or protein–protein interactions with the acylated and non-acylated N-terminal sequences.

Under physiological conditions, the native form of SP-C is palmitoylated and is closely associated with surfactant phospholipid membranes. We have shown recently that the primary sequence of this region of SP-C has an amphipathic character [27], including an intrinsic tendency to associate with phospholipid membranes [22] and interfacial films [23]. Palmitoylation, therefore, is not strictly required for primary membrane association, making it important to investigate whether this postranslational modification has other specific functions that are of potential relevance to pulmonary surfactant physiology. We have proposed that the N-terminal segment of SP-C forms an amphipathic hairpin, including a central hydrophobic β-turn that becomes even more hydrophobic upon acylation, flanked by two cationic loci [27]. The data from the present study show that bilayer insertion of this putative hydrophobic hairpin results in formation of an interdigitated-like lipid phase. In such structures, as illustrated by the cartoon in Fig. 9a, the central hydrophobic part of the hairpin could establish direct interactions with distal segments of the phospholipid acyl chains while the polar residues of the peptide hairpin could still interact with the lipid headgroups. This model would be consistent with the effect of the peptides on the chain flexibility profile of both DPPC and DPPG bilayers at temperatures below their main transition.

In a previous study, we reported the effect on membranes of a peptide designed from the N-terminal segment of SP-C but having two cysteines blocked by acetamidomethyl groups [22]. From the data available at the time we assumed that this peptide was a good mimic for the N-terminal segment of SP-C. The blocked N-terminal analog showed a strong perturbation of the fluid phase of DPPC and DPPG bilayers, without promoting interdigitated-like phases such as those described here. The present study now shows that the peptides with free or palmitoylated cysteines, unlike that with the spurious acetamidomethyl group, are well suited to intercalate within ordered-like lipids such as those potentially taking part in the P'_β phase. This feature is probably required to promote formation of interdigitated lipid regions. Fig. 5 shows maximal immobilization effects of the peptides around positions 5 and 14 of the phospholipid acyl chains, which is characteristic of an interdigitated gel phase [33,35].

Intercalation of palmitic chains attached to the N-terminal peptides could stabilize the interdigitated structure, resulting in the appearance of an increasing population of interdigitated lipids with increasing peptide/lipid ratio (seen in Figs. 6 and 7). Intercalation of peptide and phospholipid molecules to form interdigitated structures could then

inhibit the $L_\beta \rightarrow P'_\beta$ transition, maintaining maximal order of the acyl chains up to temperatures close to the main gel-to-fluid phase transition. Interestingly, a recent study has proposed that the P'_β ripple phase might consist of a coexistence of bilayered and interdigitated lipid phases [42]. Palmitoylation could then promote stable association of the N-terminal segment with interdigitated patches that might already exist in the ripple phase and promote their spatial extension. This property could be important to ensure maximal stability of DPPC-enriched domains segregated in pulmonary surfactant films at physiological temperatures [30,31]. These domains might have order and packing properties similar to those exhibited by DPPC bilayers at 37 °C, the temperature at which stabilization of interdigitated-like membrane regions by the N-terminal segment seems to be maximal. In one of the few studies published so far on lipid–protein interactions of acylated peptides, a specific effect of an acylated peptide on the P'_β ripple phase of DPPC bilayers has also been reported [43], suggesting that association with and possible stabilization of interdigitated-like regions could be a general feature favoured by palmitoylated segments of proteins. In the pure L_α phase, the lipid chains are too disordered to allow organization of well-defined interdigitated structures, and the peptides are then associated with normal bilayer structures.

Protein-attached saturated palmitic chains are particularly well suited to insert into ordered membrane structures, such as the interdigitated structures proposed here, the condensed interfacial phases reached at the end of expiration, and the liquid-ordered regions adopted by raft-like membrane domains enriched in sphingolipids and cholesterol. Several examples have been reported in the literature showing that palmitoylation may promote association of certain proteins with ordered membrane domains [44–47]. Our results also suggest that palmitoylated protein segments could contribute to nucleate ordered membrane regions, the size and stability of which still remains to be evaluated. We have observed similar qualitative effects in samples containing peptides over the whole range 1–40% protein/lipid (w/w). This includes the peptide-induced increase in $2A_{max}$, the reduction of spin–spin broadening, and the segregation of an immobilized lipid component (see ESR spectra at different peptide/lipid ratios in Figs. 4 and 7). No discontinuities were detected in the progressive effects caused by increasing peptide contents, which could have been indicative of phase transformation, e.g., micellisation in the membranes at the highest peptide/lipid ratios.

The cartoon in Fig. 9b illustrates the possible participation of the lipid/protein structures that are promoted by the palmitoylated N-terminal segment of SP-C in the activities of pulmonary surfactant at the respiratory air–liquid interface. SP-C promotes transfer of phospholipids from phospholipid bilayers to the air–liquid interface [11,18], independent of its acylation state. Simultaneous perturbation of membranes [22] and monolayers [23] by the N-terminal segment of SP-C could permit transient exposure of phospholipid molecules, hence accelerating interfacial adsorption. An SP-C-promoted transition of surfactant structures from non-interdigitated to interdigitated bilayer-like arrangements, initiated at locations where SP-C is accumulated [29,48,49], might accelerate bilayer/monolayer conversions. On the other hand, compression of SP-C-containing interfacial lipid–protein films could induce a progressive accumulation of SP-C in defined locations of the surface layers [48,50]. Sufficiently high protein density could favour formation of an ordered lipid/protein phase, where the palmitic chains of the N-terminal segment of SP-C could be intercalated with the phospholipid chains, in a manner similar to the interdigitated structure proposed here. These surface structures could be particularly favourable for initiating transitions that fold the interfacial film towards the bulk phase, and so induce formation of the surface-associated multilayered structures that are known to be promoted by SP-C [25,51]. Such multilayered structures associated with surfactant interfacial films have been demonstrated in recent years [52,53], and are now a central part of current molecular models of pulmonary surfactant action [54]. Surface-associated surfactant reservoirs would have the dual role of improving mechanical stability of highly compressed films at the end of expiration, and of promoting replenishment of the surface film with new surfactant molecules that are inserted into the interface during inspiration. Palmitoylation could prevent total dissociation of the multilayered structures from the highly packed interfacial film [24]. This possibility could explain why surfactant obtained from SP-C knock-out mice forms intrinsically unstable films [3], which could be responsible at least in part for the development of chronic respiratory problems in animals and patients whose lungs lack SP-C [4]. The ability of the SP-C N-terminal segment to maintain association with highly compressed films would be especially important at the end of exhalation, when the highest surface pressures (lowest surface tensions) must be supported.

Nevertheless, it is important to consider that the nature of the structures promoted by SP-C and its N-terminal segment in surfactant complexes that are reconstituted *in vitro* from lipid/protein or lipid/peptide organic solutions may differ from the structure and disposition of surfactant structures that are assembled *in vivo* into the lamellar bodies of pneumocytes. Very little is known about the orientation of SP-C in the membrane structures reaching the pulmonary interface. It is possible that SP-C-promoted membrane perturbations when the N-terminal segment has access to both sides of the membrane – as presumably occurs in complexes reconstituted *in vitro* – could differ from those when SP-C perturbs the membranes from a single side, either alone or in concert with other surfactant proteins. Further studies with native surfactant complexes are still required before we shall be able to model fully functional pulmonary surfactant complexes.

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