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Palmitoylation as a key factor to modulate SP-C–lipid interactions in lung surfactant membrane multilayers

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ABSTRACT

Surfactant protein C (SP-C) has been regarded as the most specific protein linked to development of mammalian lungs, and great efforts have been done to understand its structure–function relationships. Previous evidence has outlined the importance of SP-C palmitoylation to sustain the proper dynamics of lung surfactant, but the mechanism by which this posttranslational modification aids SP-C to stabilize the interfacial surfactant film along the compression–expansion breathing cycles, is still unrevealed. In this work we have compared the structure, orientation and lipid–protein interactions of a native palmitoylated SP-C with those of a non-palmitoylated recombinant SP-C (rSP-C) form in air-exposed multilayer membrane environments, by means of ATR-FTIR spectroscopy. Palmitoylation does not affect the secondary structure of the protein, which exhibits a full α-helical conformation in partly dehydrated phospholipid multilayer films. However, differences between the Amide I band of the IR spectrum of palmitoylated and non-palmitoylated proteins suggest subtle differences affecting the environment of their helical component. These differences are accompanied by differential effects on the IR bands from phospholipid phosphates, indicating that palmitoylation modulates lipid–protein interactions at the headgroup region of phospholipid layers. On the other hand, the relative dichroic absorption of polarized IR has allowed calculating that the palmitoylated protein adopts a more tilted transmembrane orientation than the non-palmitoylated SP-C, likely contributing to more compact, dehydrated and possibly stable multilayer lipid–protein films. As a whole, the behavior of multilayer films containing palmitoylated SP-C may reflect favorable structural properties for surfactant reservoirs at the air–liquid respiratory interface.

1. Introduction

Lung Surfactant associated Protein C (SP-C) has been classically considered as a marker of lung development. This protein takes part in the protein fraction of lung surfactant (LS), a complex network of lipids and proteins lining the alveolar air–liquid interface (for a review see [1]). This lipid–protein complex has evolved to reduce the work of breathing by sustaining extremely low surface tensions at the air–liquid interface and so allowing proper respiratory dynamics along the compression–expansion breathing cycles. The composition of LS has been extensively characterized, comprising more than 80% by weight of phospholipids, with an unusual proportion of disaturated species such as dipalmitoyl phosphatidylcholine (DPPC), 8–10% neutral lipids, mainly cholesterol, and 10% proteins, including specific surfactant-associated proteins SP-A, -B -C and -D. The biophysical properties exerted by LS mainly rely on its characteristic lipid composition and the presence of two of the four associated surfactant proteins, SP-B and SP-C. These proteins are absolutely required for interfacial adsorption, film stability and respreading of the surfactant material along the breathing cycles, but their specific molecular mechanism of action is still unraveled.

SP-C comprises just 35 amino acids in its mature form and it has been regarded as a lipopeptide due to its extreme hydrophobicity, its small size, and the fact that its N-terminal segment contains palmitoylated cysteines [2,3]. It results from the processing by type II pneumocytes of a 197 amino acid precursor and is secreted into the alveolar space assembled within the highly packed membranous organelles where surfactant is stored, the lamellar bodies [2]. SP-C sequence is highly conserved among species and despite that it is the most specific protein of LS with no homologous proteins found so far, its precise function is still a matter of controversy.

Structurally, SP-C consists of a poly-valine ∝-helical region and a positively-charged disordered N-terminal segment, as revealed by NMR studies in organic solvents [4]. In the context of LS bilayers, SP-C adopts a transmembrane configuration with its N-terminal segment closely associated with the membrane by the palmitic chains [5,6]. Nevertheless, some studies have shown that the N-terminal segment of SP-C has an intrinsic tendency to interact with membranes even

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when it is non-palmitoylated [7], suggesting that palmitoylation may have other role in SP-C function than merely anchor the N-terminal segment to the membrane. Besides, SP-C palmitoylation has been found to be required to sustain the proper behavior of LS films in the presence of cholesterol [8] and it has also been suggested to be necessary for interconnecting the interfacial film with the different membranous layers constituting the LS reservoir in the hypophase [9], increasing the stability of surface-associated films during breathing. For all these reasons, palmitoylation appears as a key factor modulating the proper function of SP-C.

Since Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) spectroscopy studies make possible separating protein and lipid characteristic absorption bands in the same sample, this technique appears as appropriate to provide some insights into protein–lipid interactions, secondary structure and orientation of proteins in a lipid environment [10]. ATR-FTIR is a vibrational spectroscopy technique based on the absorption of infrared light by a sample film dried on a plate made of a high reflective material. From ATR-FTIR spectra it is possible to obtain information not only on the state of lipids and proteins in the film, but also on the average configurational orientation of molecules within the film itself using polarized infrared light (for a review see [10]). Using this experimental approach the transmembrane orientation of SP-C in DPPC/phosphatidyl glycerol (PG) films has been confirmed [5,6]. Other studies have tried to analyze the effect of palmitoylation on SP-C–lipid interactions using deacylated forms of SP-C [5,11] or chemically acylated recombinant versions [12,13]. A major problem of these studies is that the treatment applied to eliminate or add the palmitic chains to the protein may alter protein structure, especially in the case of a protein with a natural tendency to form β-sheet aggregates [14].

To further analyze the nature of palmitoylation-mediated effects on SP-C–lipid interactions in a LS context, here we describe the analysis by ATR-FTIR of the native palmitoylated protein and a recombinant SP-C version (rSP-C) lacking palmitoyl chains reconstituted in three different lipid mixtures mimicking pulmonary surfactant membranes. Our results suggest that protein structure is neither modulated by the lipid composition nor palmitoylation, and that palmitoylation induces both changes in the configuration of the headgroup region of phospholipid layers and a shift in protein tilting with respect to the normal of surfactant-mimicking multilayered films.

2. Materials and methods

2.1. Materials

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3[phospho-rac-(1-glycerol)] (POPG) and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). Chloroform and methanol solvents, HPLC grade, were from LabScan (Gliwice, Poland). Trizma base, imidazole, NaCl and the rest of the reagents used for rSP-C purification were at least of molecular biology grade purchased from Sigma-Aldrich, all with a grade of purity higher than 98.5%.

2.2. Purification of native and recombinant SP-C

Native SP-C was isolated from minced porcine lungs as described elsewhere [15]. Minced lungs previously washed with saline and subjected to serial centrifugations to eliminate tissue and cell debris, were mechanically homogenized on ice and an organic extraction of the homogenized material was performed [16]. The organic extract was loaded onto a Sephadex LH-20 (GE Healthcare) equilibrated with chloroform/methanol (2:1, v/v). The protein fraction containing the hydrophobic proteins SP-B and SP-C was then pooled and applied onto a Sephadex LH-60 resin equilibrated with chloroform/methanol (1:1, v/v) acidified with a 0.5% volume of HCl (0.1 N). Purified SP-C was stored in organic solvent at −20 °C. The SP-C concentration was determined by amino acid analysis.

The recombinant SP-C was overexpressed as a fusion protein in *E. coli*, following a protocol based on the procedures described by Lukovic et al. [17] with some improvements. After induction the culture was grown for an additional period of 3 h at 37 °C. The soluble fraction of the grown cells was loaded on to a pre-packed nickel affinity chromatography column (His-Tag, GE Healthcare). Eluted protein was dialyzed o/n at 4 °C to remove imidazole and cleaved with thrombin. In order to obtain the cleaved SP-C polypeptide and to eliminate contaminant by-products, an organic extract of the cleaved mixture was performed [16] and chromatographed through the LH-20 column. To prevent protein aggregation during concentration and manipulation, POPC (5:1 lipid-to-protein ratio, w/w) was added to the cleaved mixture before being concentrated and applied onto the chromatography. The final protein is obtained again free of lipid as it elutes from LH-20. Protein purity was assessed by SDS-PAGE and the concentration was determined by amino acid analysis.

2.3. Reconstitution of lipid and lipid/protein samples

The main lipid mixture used in our study as LS membrane model consists of DPPC/POPC/POPG (50:25:15, w/w/w), to simulate physiological proportions of saturated/unsaturated and zwitterionic/anionic phospholipids found in most surfactants. To evaluate the effect of phospholipids on protein structure and orientation as well as the effect of the protein on the phospholipid behavior, samples containing 7% by weight of either native palmitoylated or recombinant non-palmitoylated SP-C were prepared.

Lipid and lipid/protein samples were prepared by mixing the appropriate amounts of protein and lipids in chloroform/methanol (2:1 v/v), and then drying the samples under a stream of nitrogen and 2 h in a SpeedVac Concentrator System (Thermo Scientific). Thereafter, multilamellar vesicles were obtained by resuspending the dry lipid or lipid–protein films to a final phospholipid concentration of 10 mg/ml in sample buffer (5 mM Tris, 150 mM NaCl pH 7) at 50 °C for 1 h with intermittent shacking. To increase sample concentration and eliminate salt excess, the samples were centrifuged 1 h at 4 °C and 4/5 of the supernatant was removed. The samples were then kept at 4 °C and resuspended prior to measurements.

2.4. Acquisition of ATR-FTIR spectra

The ATR-FTIR spectra were recorded on a Bruker IFS66 FTIR spectrophotometer (Ettlingen, Germany) equipped with a liquid nitrogen mercury cadmium telluride detector (MCT) in the double-sided, forward–backward mode at 2 cm^{-1} resolution and an aperture of 3.5 mm. The spectrometer was continuously purged with dry air. The internal reflection element was a germanium ATR plate with an aperture angle of 45° yielding 25 internal reflections. To improve signal/noise ratio, 256 accumulations were collected for each measurement. For polarization experiments, a thallium bromoiodiode wire grid polarizer (KRS-5, Specac) was positioned. Measurements were carried out at 21, 25 and 37 °C. Temperature control was achieved by a thermostatized internal reflectance element support connected to a water bath (Haake DC30 from Thermo Fisher Scientific Inc., Waltham, MS).

The recorded spectra were corrected for the atmospheric water absorbance interferences by subtracting a reference atmospheric water spectrum. The subtraction coefficient was computed as the ratio of the atmospheric water band in the range 1562–1555 cm^{-1} on the sample spectrum with respect to the reference spectrum. The spectra were then smoothed by apodization of a 4 cm^{-1} Gaussian line shape.

To prepare the samples, oriented lipid multilayers were formed on the surface of the germanium crystal by a slow evaporation of the lipid or lipid/protein aqueous suspensions under a nitrogen stream [18]. Parallel orientation of lipid multilayers with respect to the ATR

crystal surface was confirmed upon observation of the dichroic spectra. All experiments were carried out in films containing 35 μg of protein with a protein/lipid ratio 1/7 (w/w).

2.5. Secondary structure determination

The shape and intensity of the Amide I band is a sum of the contributions of all the secondary structure components of the protein or mixture of proteins present in the samples. To obtain the proportion of each particular secondary structure from the original spectrum, a deconvolution method is required [19]. To avoid possible interferences due to the analysis method, the spectra were also directly compared upon normalization with respect to amide I total intensity and a subtraction spectra (SP-C − rSP-C) was obtained.

2.6. Molecular orientation

The determination of protein molecular orientation by infrared ATR spectroscopy has been reviewed by Goormaghtigh et al. [10]. The spectra were recorded with a parallel or perpendicular polarized incident light with respect to the plane defined by the ATR crystal. The dichroic ratio, R^{ATR}, is defined as the ratio of the absorption band recorded for the perpendicular polarization ($A^{\perp}$) with respect to the parallel polarization ($A^{\parallel}$):

$$R^{ATR} = A^{\perp}/A^{\parallel}$$

In ATR, the dichroic ratio for an isotropic sample is different from the unity and is usually evaluated as the mean dichroism of the two lipid ester ν(C═O) bonds absorbing between 1762 and 1716 cm^{-1}. The mean orientation of the molecular axis with respect to the perpendicular of the ATR plate was estimated as described before [10]. There is no consensus in the determination of an appropriate value for the orientation of amide I dipole in the α-helical structure; nevertheless a value of 27° was used for the calculations [10].

2.7. Determination of band position

Accurate band position (better than 0.1 cm^{-1}) was obtained by fitting a series of 11 data points, i.e. 5 cm^{-1} before the approximate maximum and 5 cm^{-1} after, by a third order polynomial and finding the roots of its derivative. It needs to be emphasized that this level of accuracy is much better than the nominal resolution of the spectrometer, which was set at 2 cm^{-1} and than the encoding of the data (every 1 cm^{-1}).

3. Results

3.1. Protein structure

To analyze whether protein structure was altered by the lipid environment or palmitoylation, the ATR-IR spectra of palmitoylated and non-palmitoylated SP-C were obtained in films prepared from suspensions of the proteins in different phospholipid mixtures. The mixture DPPC:POPC:POPG (50:25:15, w/w/w) was used as a model capturing the main properties of LS in terms of the combination of saturated/unsaturated and zwitterionic/anionic phospholipid species. The other two lipid systems tested were DPPC:POPC (50:40, w/w) and POPC:POPG (75:15, w/w) in order to define the importance of the charge and lipid phase coexistence on protein–lipid interactions, respectively.

Fig. 1 compares the ATR-FTIR spectrum of SP-C and rSP-C inserted into DPPC/POPC/POPG membranes, once normalized with respect to their Amide I band. The figure also includes a difference spectrum, which reveals the apparent shift of Amide I contribution of palmitoylated with respect to the non-palmitoylated form of SP-C. Amide I and II bands of SP-C and rSP-C appeared centered around 1657 cm^{-1} and 1543 cm^{-1}, respectively. The narrow shape of these bands and their frequencies' maxima are consistent with a predominantly alpha helical structure as it has been previously described for these proteins [4,17]. Estimations of the secondary structure for the two proteins calculated more than 80% α-helix and no differences between them. However, the SP-C − rSP-C difference spectrum revealed an upward band deviation in the amide I wavenumbers, which increases in intensity and decreases in frequency with temperature.

Fig. 2 compares the IR spectra and the difference between palmitoylated and non-palmitoylated proteins with respect to Amide I contribution for the other two lipid environments assessed, DPPC/POPC and POPC/POPG, at three different temperatures. The shift in Amide I wavenumber in palmitoylated vs. non-palmitoylated protein is apparent in DPPC/POPC multilayers but not so much in the purely unsaturated phospholipid films. This difference is slight but consistent in the different experiments, and can be associated with a slightly smaller frequency of vibration for amides of the palmitoylated protein.

Hydrogen-bonding is known to reduce the frequency of stretching vibrations [20], indicating that this shift could be associated with differences in the access of water to different segments of the headgroup region of phospholipid bilayers, caused either by differences in the extent of perturbation of the membrane by the two proteins or by differences in the orientation, the extent of insertion or the tilt of the two proteins in the membranes.

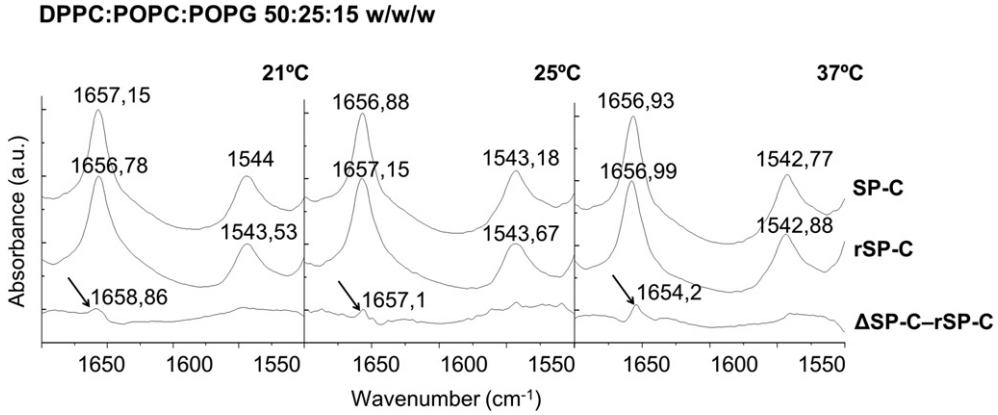

Fig. 1. SP-C and rSP-C structure in DPPC:POPC:POPG. ATR-FTIR spectra of SP-C and rSP-C reconstituted in DPPC:POPC:POPG (50:25:15, w/w/w) multilayers, at 21 °C, 25 °C and 37 °C. Frequency maxima for amide I (1700–1600 cm^{-1}) and amide II (1600–1500 cm^{-1}) bands are shown. Differences between the two proteins are illustrated in the subtraction spectra SP-C − rSP-C below, with the main difference marked by an arrow. Both spectra were rescaled to the same intensity for the amide I band (1700–1600 cm^{-1}).

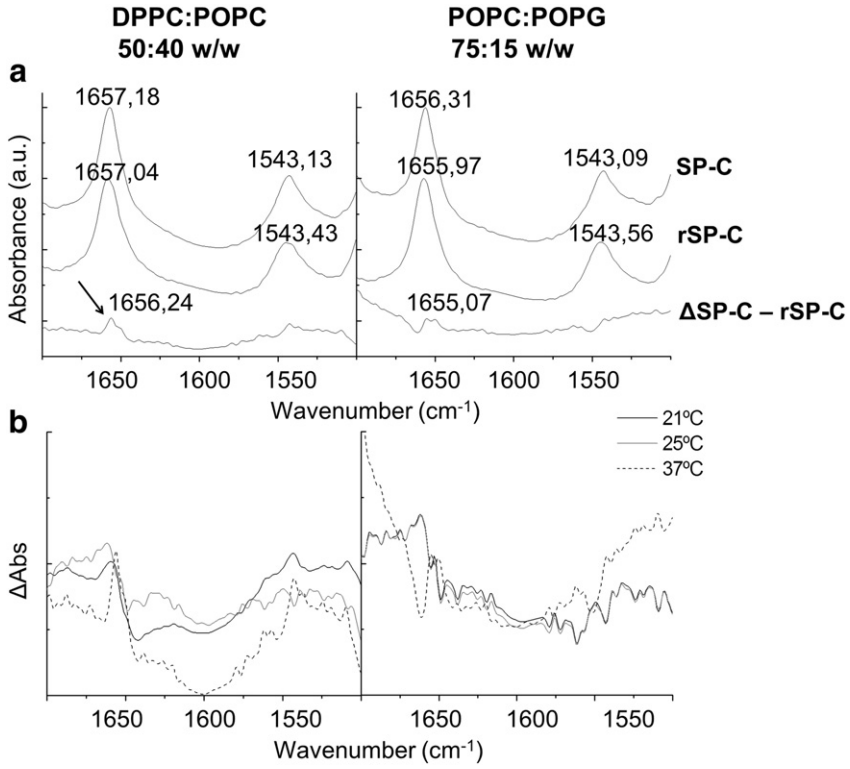

Fig. 2. Structure of SP-C and rSP-C in different lipid environments. (a) ATR-FTIR spectra of proteins SP-C and rSP-C reconstituted in DPPC:POPC (50:40, w/w) or POPC:POPG (75:15, w/w) membrane multilayers, at 37 °C. Frequency maxima for amide I (1700–1600 cm^{-1}) and amide II (1600–1500 cm^{-1}) bands are shown. SP-C − rSP-C represents the subtraction spectra. (b) Subtraction spectra SP-C − rSP-C at 21 °C (black), 25 °C (gray) and 37 °C (dashed line), for each of the two lipid compositions. All spectra were rescaled to the same intensity.

3.2. Lipid–protein interactions

To assess whether SP-C and rSP-C could exhibit a different extent of perturbation at the different lipid systems studied, we have monitored the thermotropic profile of the phospholipids by following the shift of the frequency associated with CH_2 stretching vibrations. These bands are sensitive to the lipid chain *trans*/*gauche* isomerizations leading to temperature-dependent frequency changes. An increase in temperature generates a vibrational band toward higher wavenumbers, which can be used to compare the thermotropic profiles of the phospholipid layers in the absence or in the presence of either palmitoylated or non-palmitoylated protein.

Fig. 3 shows the thermotropic behavior of the three lipid systems studied, in the absence and in the presence of the native or the recombinant SP-C variants. In none of the lipid compositions studied there is a clear effect attributable to the protein insertion into the membrane in the range of temperatures analyzed. Methylene asymmetric stretching frequencies (2900–2950 cm^{-1}) overlap for the samples containing or not SP-C or rSP-C at the three temperatures tested, suggesting that neither lipid composition nor palmitoylation are critical factors to modulate lipid–protein interactions at the multilayer membrane films, at least as sensed by the acyl chains under the conditions used for this study.

To gain further insight into the characterization of SP-C–lipid interactions, we focused on the differences observed in the polar region of the lipid spectra. Phosphate-associated vibration bands can provide information about the superficial interactions in the membrane that modulate both their intensity and frequency [21]. Fig. 4a illustrates the effect of the palmitoylated or non-palmitoylated SP-C on the phosphate-associated vibration bands for the three different lipid systems studied. The largest differences are observed in the phosphate asymmetric and symmetric stretching region in the presence of the palmitoylated SP-C

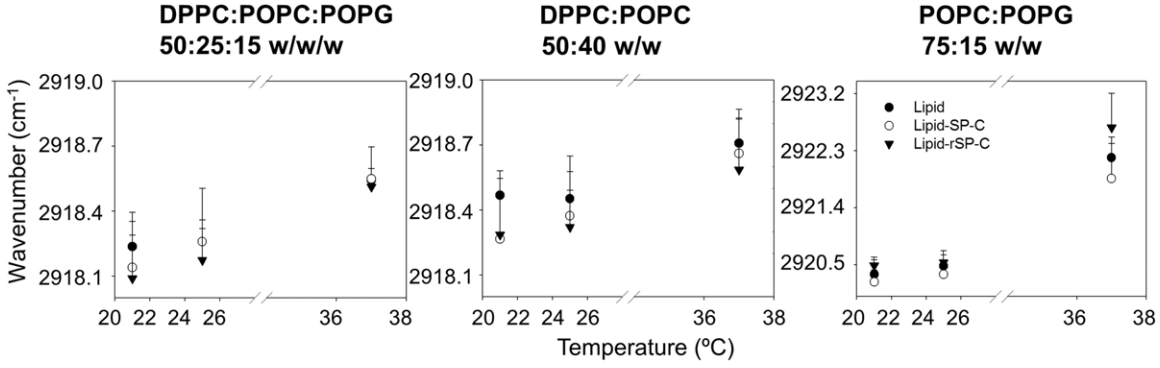

Fig. 3. Effect of SP-C and rSP-C on the thermotropic profile of different membrane multilayer systems. The thermotropic profiles of the three different lipid systems assessed, in the absence or in the presence of proteins SP-C or rSP-C, are monitored by the position of the $\nu_{as}(CH_2)$ absorption band. The profiles show the effect attributable to protein insertion into the membrane for each temperature studied.

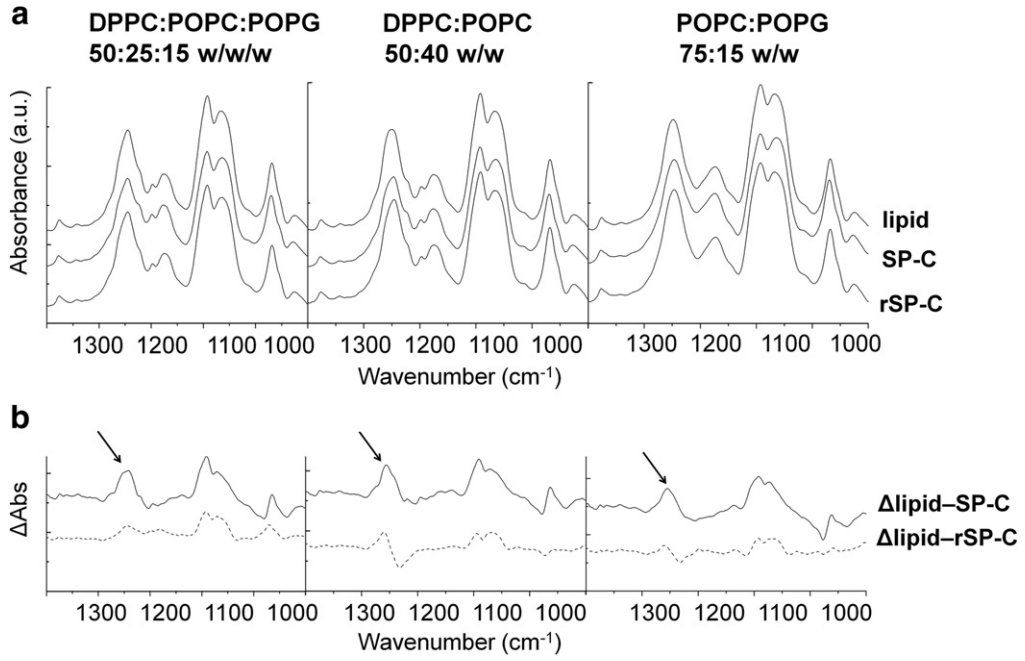

Fig. 4. Effect of SP-C on phosphate vibrations in different lipid systems. (a) Spectra corresponding to the phosphate vibration region (1400–900 cm^{-1}) are shown for the three different lipid systems assessed, in the absence or in the presence of proteins SP-C or rSP-C, at 37 °C. (b) Subtraction spectra of pure lipid–lipid/SP-C (black line) and lipid–lipid/rSP-C (dashed line). The main difference, introduced mainly by palmitoylated SP-C is marked with a black arrow.

protein compared to the samples containing non-palmitoylated SP-C. For the surfactant mimicking synthetic mixture, DPPC:POPC:POPG (50:25:15, w/w/w), the differences are mainly observed on peak intensity, which decreases to a lower level when the incorporated protein is the non-palmitoylated SP-C. For the other two lipid systems, the differences are also more conspicuous in the presence of the palmitoylated protein, although there are also some variations on the frequency of the vibrations. These alterations could be associated with a different extent of perturbation of the headgroup region of the membrane multilayers by the N-terminal segment of the protein.

3.3. Molecular orientation

The molecular orientation of SP-C and rSP-C in the three lipid systems studied, as estimated by the dichroic absorption ratio of polarized IR, is summarized in Table 1. The different effects can be observed on the calculated tilting of the protein depending on the temperature, palmitoylation or lipid composition. A slight increase on the tilting angle was found at higher temperatures for both proteins in all compositions tested, which could be consistent with the assumption that disordering on the lipid membranes at higher temperatures decreases membrane thickness causing a hydrophobic mismatch resolved by the tilting of the helix [22,23].

On the other hand, palmitoylation seems to induce a consistent trend toward a higher tilting degree of the protein in the membranes for most of the cases analyzed. Palmitoylation-induced anchoring of SP-C to the membrane may induce a deeper embedment of the N-terminal end of the protein into the polar region of the bilayer pulling out the helix to an increased tilting angle with respect to the membrane normal. This is consistent with the fact that depalmitoylation induced a more perpendicular orientation of the protein when it is reconstituted in lipid bilayers as reviewed in [3]. Nevertheless, the effect of palmitoylation cannot be analyzed independently of lipid composition. POPG-containing systems induced a more tilted protein configuration for both SP-C and rSP-C at 21 °C than estimated for the purely zwitterionic system. This could indicate again a closer association of the N-terminal segment with the membrane in this case due to electrostatic interactions, as it has been previously described [7]. The positively charged N-terminal end of both proteins would then be interacting with the negative charge of POPG forcing again the helix to adopt a less perpendicular orientation.

Table 1
Tilting angles for SP-C and rSP-C proteins in the different lipid environments.

		DPPC:POPC:POPG 50:25:15 w/w/w	DPPC:POPC 50:40 w/w	POPC:POPG 75:15 w/w
21 °C	SP-C	25° ± 5°	20° ± 5°	25° ± 5°
	rSP-C	20° ± 5°	17.5° ± 2.5°	22.5° ± 5°
25 °C	SP-C	25° ± 5°	20° ± 5°	25° ± 5°
	rSP-C	20° ± 5°	17.5° ± 2.5°	27.5° ± 5°
37 °C	SP-C	30° ± 5°	22.5° ± 2.5°	37.5° ± 2.5°
	rSP-C	22.5° ± 2.5°	32.5° ± 2.5°	37.5° ± 2.5°

4. Discussion

Palmitoylation is thought to be one of the main features associated with the functional activity of surfactant protein SP-C [8,11], contributing to define the optimal conditions for the re-spreading ability of LS during inspiration [24,25]. This has been related with the ability of the palmitoylated protein to maintain association of surfactant bilayers with the interfacial film, forming what has been called an interfacial surfactant reservoir. In the current work, we have studied the structure, lipid–protein interactions and orientation of palmitoylated and non-palmitoylated versions of SP-C in partly dehydrated multilayer films formed upon deposition and dryness of lipid/protein suspensions on the surface of the germanium crystal of an ATR-IR device. The oriented multilayers formed in the ATR-FTIR approach retain solvation water molecules, thus, maintaining potential lipid–protein and lipid–lipid interactions mediated by the presence of water. Thus, we propose that this air-exposed multilayered structure somehow mimics the organization of lipids and proteins at the multilayer reservoir of surfactant, once it has been formed upon exclusion from the interface during compression and/or upon incorporation of newly adsorbing lipid–protein complexes. Our results suggest that palmitoylation is important to modulate the proper configuration of the protein in these LS mimicking multilayers; how this configuration could be related to the function of the protein has to be analyzed carefully.

In terms of protein structure, several studies have previously addressed the effect of SP-C palmitoylation with controversial results. Some of them have suggested that depalmitoylation induces a loss of alpha helical structure increasing the chance of protein aggregation [5,11,26], while others have pointed out that it does not alter the α-helical structure and may even favor it [27,28]. SP-C has a strong tendency to aggregate even in non-polar solvents [29], particularly when it is free of lipids. For this reason, some of the chemical treatments used in those previous studies to add or remove the acyl chains could be the indirect or direct cause of the alteration in the protein structure, rather than the absence or presence of the chains themselves. Our study suggests that palmitoylation does not affect much the secondary structure of the protein as the infrared difference spectrum shows no big differences between SP-C and rSP-C proteins in the different lipid systems studied, which is consistent with the results found in a previous study [27]. It could well be that the structure of the protein is particularly stabilized at its maximal α-helical conformation in the partly dehydrated, possibly highly packed, multilayered films, and this could be the reason why our estimations of that secondary structure yield at least 80% α-helix, for the two proteins in all the lipid environments, though a subtle but consistent difference was revealed as a shift of the α-helical wavenumbers from the difference spectra in the amide I region of palmitoylated and non-palmitoylated SP-C. Stretching vibrations are influenced by the polarity of the environment of any defined IR chromophore. Thus, differences in the presence of water molecules that could form hydrogen bonds with our molecule of study could explain such a decrease of the frequency of stretching vibrations. Taking into account that the energy of amide I band comes principally from *ν*C═O bond [30], a change of environment polarity could be behind the shift in the vibration frequency of this bond. On the other hand, SP-C has been recently found to promote phospholipid vesicle instability, conferring to membranes a highly dynamic character, which leads to the formation of evaginations from giant vesicles [31]. This fact could be attributed to protein-mediated induction of curvature, with important perturbations at the headgroup region of membranes. In the dehydrated multilayered films, as they are formed at the air-exposed surface, a formation of curved membranes could be prevented, and protein perturbation induced at intermembrane surfaces could end in larger packing defects and deeper water penetration. Protein-promoted perturbations may be also important to facilitate intermembrane topological connections and efficient transfer of surface active lipid molecules through the whole surface film. From our results, we speculate that palmitoylation could generate a deeper insertion of the N-terminal segment of SP-C into the headgroup region of the lipid layers, producing larger packing defects and a local polarity increase in the membrane. In this sense, and considering that palmitoylated SP-C is also on average more tilted into membranes than the non-palmitoylated form, the more external N-terminal end of the α-helix would sense this change of polarity varying its frequency of vibration to lower wavenumbers. This effect is particularly observed when the lipid composition includes saturated phospholipids, suggesting a modulating effect of membrane composition. SP-C has been found to locate preferentially into liquid-disordered phases in membranes [32] and liquid-expanded regions in interfacial films [33,34], but accumulating at the boundaries between ordered and disordered regions in membranes and monolayers exhibiting phase coexistence [35]. We propose that SP-C, or at least its palmitoylated N-terminal segment, could be located at the edge of the domains formed by DPPC, where SP-C-induced perturbation could be maximal. In our experiments the difference in the amide I spectra of palmitoylated and non-palmitoylated SP-C increases while approaching 37 °C in those systems whose melting temperature is in the range of physiological temperatures. With the increasing temperature, the membranes reach a higher dynamic character, including more frequent and larger molecular fluctuations, deeper water penetration and a possibly larger difference in water accessibility of the N-terminal end of SP-C helix. If palmitoylation is at least in part responsible for the association of SP-C with the boundaries of DPPC-enriched ordered lipid domains, differences in perturbations induced between palmitoylated and non-palmitoylated SP-C would be further accentuated as it is the case. The stronger effect of the palmitoylated protein to perturb the contribution of phosphates to the IR spectra would be consistent with the changes promoted by the acylated segment of the protein on the organization, packing and polarity of the headgroup region of the lipid/protein multilayers. The effect of either palmitoylated or non-palmitoylated protein on the thermotropic profile sensed by the acyl chains is more limited, indicating that the effect of palmitoylation is somehow focused to that headgroup level. At the closely apposed intermembrane surfaces of the multilayered surfactant reservoir, SP-C promoted perturbations could be particularly important to introduce a dynamic factor that may contribute to the efficient mobilization of surface active molecules between the reservoir and the interface. This effect could be more important the more dehydrated and closely packed the reservoir is.

The study of SP-C orientation in membranes has been addressed before [5,6,36]. Most of these studies have indicated an orientation for the alpha helix almost parallel to the acyl chains. In the present work we have observed different grades of protein tilting depending on the lipid system studied and on the palmitoylation state. Discrepancies between the previous values and those obtained in the current study could come from the assumption of an order parameter (S) of 1 for our determinations, which was compatible with our results and theoretical values. Therefore, the angles discussed here correspond to maximum tilts, although it is not possible to discard the existence of disorder sources that could shift the values to more perpendicular orientations.

Temperature seemed to have some effect on protein orientation, increasing slightly the tilting angles toward higher values. This effect could be originated in a more pronounced hydrophobic mismatch when membrane thickness decreases at higher temperature, particularly when temperatures above Tm are reached and the phase transition occurs, as it may be the case for the lipid mixtures containing DPPC.

The results presented here have shown that palmitoylated SP-C may alter the polar region of the membranes at a larger extent than rSP-C, and that the helix may be suffering the effect of a more polar environment caused by a deeper embedment of the N-terminal segment into the headgroup region of the phospholipid layers when compared to the effect for the non-palmitoylated protein. These observations are consistent with our findings on protein orientation. When the protein is inserted into multilayered films made of the ternary system mimicking LS composition, palmitoylation seems to roughly induce a 5° more pronounced tilting angle of the transmembrane helix with respect to the normal of the layers compared to that of non-palmitoylated rSP-C. We are aware that the magnitude of this increase in the tilting angle is close to the limit of significance defined by the calculation method, but we still see it as relevant considering that in most experiments this trend is maintained in the same direction. Such palmitoylation-promoted increase on helical tilting is not so clear in the system containing no DPPC, suggesting that for palmitoylation to cause a more pronounced tilt, a context of thicker membranes or of membranes sustaining a coexistence of ordered/disordered phases could be required. It is important to mention that for those systems containing POPG, the calculated tilting angles were higher for both proteins when compared to those in the purely zwitterionic DPPC:POPC system, supporting the idea that electrostatic interactions between the N-terminal segment of the proteins and the anionic phospholipid also contribute to restricting SP-C mobility and modulate its orientation.

Finally, one has to consider the particular context imposed by the insertion of the protein into the partly dehydrated multilayer phospholipid films studied here. The cartoon in Fig. 5 presents a model illustrating how selective protein–lipid interactions and differential tilting could define the configuration of SP-C in single membrane and in multilayer membrane arrangements. It has been proposed that the palmitoylated segment of SP-C might sustain connection between the different layers in the surface-associated surfactant reservoir. This could be particularly

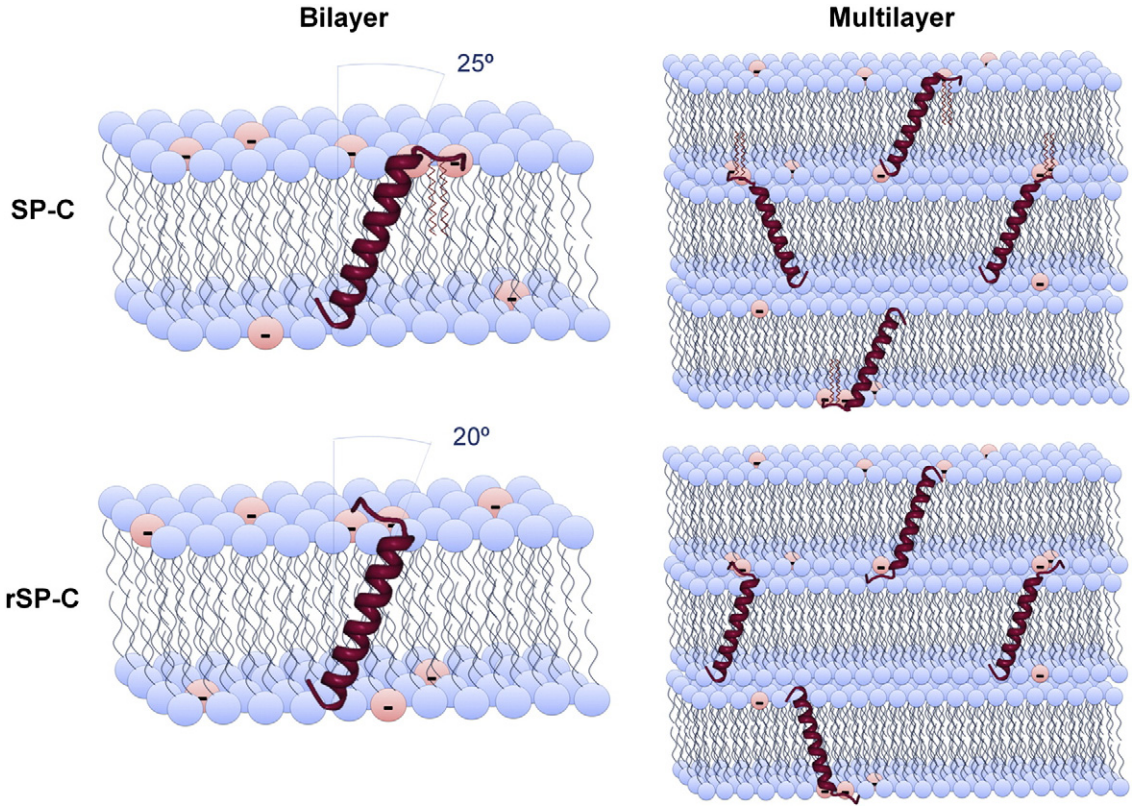

Fig. 5. Model for the role of palmitoylation of SP-C and the presence of anionic phospholipids in lipid bilayers and multilayers.

important during the formation of this reservoir, upon SP-C-promoted exclusion of lipids and proteins during compression of the interfacial film at expiration. Palmitoylation-promoted intermembrane contacts might maintain a low hydration level of the reservoir, which could facilitate re-spreading during inspiration. The partly dehydrated multilayered films studied in the current work could mimic such operative surface reservoir, with the revealed particular lipid–protein interactions and tilting induced by the palmitoylated protein being possible signatures of a functional reservoir highly competent to re-spread into the interface and re-establish a functional surface-active film. Still, it is important to consider that the connection of the surface multilayer reservoir with the different surfactant structures arriving from the subphase could introduce a dynamic factor that is difficult to mimic in the ATR approach. It remains a major challenge to extend this study to the determination of the molecular organization of lipids and proteins in surfactant films in situ, where the interfacial compression–expansion dynamics imposes additional constraints.

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References

[1] E. Lopez-Rodriguez, J. Perez-Gil, Structure–function relationships in pulmonary surfactant membranes: from biophysics to therapy, Biochim. Biophys. Acta 1838 (6) (2014) 1568–1585.
[2] A. ten Brinke, L.M. van Golde, J.J. Batenburg, Palmitoylation and processing of the lipopeptide surfactant protein C, Biochim. Biophys. Acta 1583 (3) (2002) 253–265.
[3] J. Johansson, Structure and properties of surfactant protein C, Biochim. Biophys. Acta 1408 (2–3) (1998) 161–172.
[4] J. Johansson, et al., The NMR structure of the pulmonary surfactant-associated polypeptide SP-C in an apolar solvent contains a valyl-rich alpha-helix, Biochemistry 33 (19) (1994) 6015–6023.
[5] G. Vandenbussche, et al., Structure and orientation of the surfactant-associated protein C in a lipid bilayer, Eur. J. Biochem. 203 (1–2) (1992) 201–209.
[6] B. Pastrana, A.J. Mautone, R. Mendelsohn, Fourier transform infrared studies of secondary structure and orientation of pulmonary surfactant SP-C and its effect on the dynamic surface properties of phospholipids, Biochemistry 30 (41) (1991) 10058–10064.
[7] I. Plasencia, et al., The N-terminal segment of pulmonary surfactant lipopeptide SP-C has intrinsic propensity to interact with and perturb phospholipid bilayers, Biochem. J. 377 (Pt 1) (2004) 183–193.
[8] F. Baumgart, et al., Palmitoylation of pulmonary surfactant protein SP-C is critical for its functional cooperation with SP-B to sustain compression/expansion dynamics in cholesterol-containing surfactant films, Biophys. J. 99 (10) (2010) 3234–3243.
[9] P. Na Nakorn, et al., Surfactant protein C and lung function: new insights into the role of alpha-helical length and palmitoylation, Eur. Biophys. J. 36 (4–5) (2007) 477–489.
[10] E. Goormaghtigh, V. Raussens, J.M. Ruysschaert, Attenuated total reflection infrared spectroscopy of proteins and lipids in biological membranes, Biochim. Biophys. Acta 1422 (2) (1999) 105–185.
[11] Z. Wang, et al., Acylation of pulmonary surfactant protein-C is required for its optimal surface active interactions with phospholipids, J. Biol. Chem. 271 (32) (1996) 19104–19109.
[12] S. Hawgood, et al., Lung function in premature rabbits treated with recombinant human surfactant protein-C, Am. J. Respir. Crit. Care Med. 154 (2 Pt 1) (1996) 484–490.
[13] L.A. Creuwels, et al., Effect of acylation on structure and function of surfactant protein C at the air–liquid interface, J. Biol. Chem. 268 (35) (1993) 26752–26758.
[14] M. Gustafsson, et al., Amyloid fibril formation by pulmonary surfactant protein C, FEBS Lett. 464 (3) (1999) 138–142.
[15] J. Perez-Gil, A. Cruz, C. Casals, Solubility of hydrophobic surfactant proteins in organic solvent/water mixtures. Structural studies on SP-B and SP-C in aqueous organic solvents and lipids, Biochim. Biophys. Acta 1168 (3) (1993) 261–270.
[16] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (8) (1959) 911–917.
[17] D. Lukovic, et al., Production and characterisation of recombinant forms of human pulmonary surfactant protein C (SP-C): structure and surface activity, Biochim. Biophys. Acta 1758 (4) (2006) 509–518.
[18] U.P. Fringeli, H.H. Gunthard, Infrared membrane spectroscopy, Mol. Biol. Biochem. Biophys. 31 (1981) 270–332.

[19] D.M. Byler, H. Susi, Examination of the secondary structure of proteins by deconvolved FTIR spectra, Biopolymers 25 (3) (1986) 469–487.
[20] N.B. Colthup, L.H. Daly, S.E. Wiberley, Introduction to Infrared and Raman Spectroscopy, 2nd ed. Academic Press, New York, 1975.
[21] W. Hübner, A. Blume, Interactions at the lipid–water interface, Chem. Phys. Lipids 96 (1998) 99–123.
[22] Z.V. Leonenko, et al., Investigation of temperature-induced phase transitions in DOPC and DPPC phospholipid bilayers using temperature-controlled scanning force microscopy, Biophys. J. 86 (6) (2004) 3783–3793.
[23] J.A. Killian, Hydrophobic mismatch between proteins and lipids in membranes, Biochim. Biophys. Acta 1376 (3) (1998) 401–415.
[24] M. Gustafsson, et al., Palmitoylation of a pulmonary surfactant protein C analogue affects the surface associated lipid reservoir and film stability, Biochim. Biophys. Acta 1466 (1–2) (2000) 169–178.
[25] A. Kramer, et al., Distribution of the surfactant-associated protein C within a lung surfactant model film investigated by near-field optical microscopy, Biophys. J. 78 (1) (2000) 458–465.
[26] R.A. Dluhy, et al., Deacylated pulmonary surfactant protein SP-C transforms from alpha-helical to amyloid fibril structure via a pH-dependent mechanism: an infrared structural investigation, Biophys. J. 85 (4) (2003) 2417–2429.
[27] E.J. Veldhuizen, et al., Production of surfactant protein C in the baculovirus expression system: the information required for correct folding and palmitoylation of SP-C is contained within the mature sequence, Biochim. Biophys. Acta 1416 (1–2) (1999) 295–308.
[28] B. Luy, et al., Structure and potential C-terminal dimerization of a recombinant mutant of surfactant-associated protein C in chloroform/methanol, Eur. J. Biochem. 271 (11) (2004) 2076–2085.
[29] T. Szyperski, et al., Pulmonary surfactant-associated polypeptide C in a mixed organic solvent transforms from a monomeric alpha-helical state into insoluble beta-sheet aggregates, Protein Sci. 7 (12) (1998) 2533–2540.
[30] A. Barth, Infrared spectroscopy of proteins, Biochim. Biophys. Acta 1767 (9) (2007) 1073–1101.
[31] E. Parra, et al., A combined action of pulmonary surfactant proteins SP-B and SP-C modulates permeability and dynamics of phospholipid membranes, Biochem. J. 438 (3) (2011) 555–564.
[32] J. Bernardino de la Serna, et al., Cholesterol rules: direct observation of the coexistence of two fluid phases in native pulmonary surfactant membranes at physiological temperatures, J. Biol. Chem. 279 (39) (2004) 40715–40722.
[33] K. Nag, et al., Fluorescently labeled pulmonary surfactant protein C in spread phospholipid monolayers, Biophys. J. 71 (1) (1996) 246–256.
[34] K. Nag, et al., Combinations of fluorescently labeled pulmonary surfactant proteins SP-B and SP-C in phospholipid films, Biophys. J. 72 (6) (1997) 2638–2650.
[35] R. Wustneck, et al., Interfacial properties of pulmonary surfactant layers, Adv. Colloid Interf. Sci. 117 (1–3) (2005) 33–58.
[36] L. Gomez-Gil, J. Perez-Gil, E. Goormaghtigh, Cholesterol modulates the exposure and orientation of pulmonary surfactant protein SP-C in model surfactant membranes, Biochim. Biophys. Acta 1788 (9) (2009) 1907–1915.