Available online at www.sciencedirect.com

SCIENCE DIRECT

Biochimica et Biophysica Acta 1713 (2005) 118 – 128

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

http://www.elsevier.com/locate/bba

Interaction of the N-terminal segment of pulmonary surfactant protein SP-C with interfacial phospholipid films

Ines Plasencia[a,1], Kevin M.W. Keough[b], Jesus Perez-Gil[a,*]

[a]*Departamento de Bioquímica, Facultad de Biología, Universidad Complutense, 28040 Madrid, Spain*
[b]*Departments of Biochemistry and Pediatrics, Memorial University of Newfoundland, St. John's, Canada A1B 3X9*

Received 4 April 2005; received in revised form 10 June 2005; accepted 10 June 2005
Available online 23 June 2005

Abstract

Pulmonary surfactant protein SP-C is a 35-residue polypeptide composed of a hydrophobic transmembrane alpha-helix and a polycationic, palmitoylated-cysteine containing N-terminal segment. This segment is likely the only structural motif the protein projects out of the bilayer in which SP-C is inserted and is therefore a candidate motif to participate in interactions with other bilayers or monolayers. In the present work, we have detected intrinsic ability of a peptide based on the sequence of the N-terminal segment of SP-C to interact and insert spontaneously into preformed zwitterionic or anionic phospholipid monolayers. The peptide expands the π–A compression isotherms of interfacial phospholipid/peptide films, and perturbs the lipid packing of phospholipid films during compression-driven liquid-expanded to liquid-condensed lateral transitions, as observed by epifluorescence microscopy. These results demonstrate that the sequence of the SP-C N-terminal region has intrinsic ability to interact with, insert into, and perturb the structure of zwitterionic and anionic phospholipid films, even in the absence of the palmitic chains attached to this segment in the native protein. This effect has been related with the ability of SP-C to facilitate reinsertion of surface active lipid molecules into the lung interface during respiratory compression–expansion cycling.

Keywords: Pulmonary surfactant; SP-C; Monolayer; Air–liquid interface; Lipid–protein interaction

1. Introduction

SP-C, one of the specific proteins assembled into the lipid–protein complex of pulmonary surfactant, is a lipopeptide expressed specifically in lung tissue, synthesized and secreted by the type II pneumocytes of the alveolar epithelium.

The main function of pulmonary surfactant is the stabilization of the bronchoalveolar structure of lungs through modulation of the surface tension at the respiratory air–liquid interface [1]. Phospholipids, especially dipalmitoylphosphatidylcholine (DPPC), play the major biophysical role in surfactant, due to their unique physicochemical properties that allow formation of stable interfacial monomolecular films able to achieve and sustain very low surface tensions. Other lipid components such as the anionic phospholipid phosphatidylglycerol (PG) and specific surfactant-associated proteins are required for surfactant to undergo complex and dynamic structural transformations in the airways after its secretion from pneumocytes [2,3]. Such transformations have the ultimate effect of facilitating rapid movement of surface active species into the air–liquid interface. Surfactant interfacial films are ultimately composed of the phospholipid-enriched surface active monolayer and some associated bilayers that have been proposed to act as a sort of surface-associated reservoir of surfactant material [4,5].

Of the four specific surfactant-associated polypeptides, SP-B and SP-C are very hydrophobic and are considered essential in promoting transfer of lipids into the air–liquid interface and modulating the physical properties of the surfactant film under the dynamic conditions imposed by

* Corresponding author. Tel.: +34 91 3944994; fax: +34 91 3944672.
E-mail address: jpg@bbm1.ucm.es (J. Perez-Gil).
[1] Present address: MEMPHYS—Center for Biomembrane Physics, Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark.

0005-2736/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2005.06.002

the respiratory cycle [6]. However, the precise role of SP-C in the respiratory physiology has still to be fully defined. This protein is not strictly required to initiate respiratory function at birth as seen in animals where expression of the SP-C gene has been genetically deactivated [7,8]. However, the lungs from these SP-C knock-out animals seem to be intrinsically unstable. 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 [9–11]. In vitro, SP-C has been shown to promote phospholipid interfacial adsorption and to stabilize interfacial monolayers subjected to dynamic compression–expansion cycling [12,13]. SP-C seems also to participate in the formation of the surface-attached surfactant reservoir providing a continuous supply of surface-active molecules to replenish the interfacial film during successive respiratory cycles [14,15].

SP-C is a 35-amino acid polypeptide, with a molecular mass of 4 kDa [16]. Its three-dimensional structure has been determined by NMR in chloroform/methanol solutions, where it is completely soluble because of its extreme hydrophobicity [17]. The protein consists of a regular hydrophobic alpha-helix comprising 23 residues and a more polar 10-residue N-terminal segment, which has no defined conformation in organic solvents or in detergent micelles [17,18] and contains stoichiometrically palmitoylated vicinal cysteines [19]. The C-terminal α-helix adopts a transmembrane orientation in phospholipid bilayers [20,21] with the N-terminal segment exposed to the aqueous phase [22,23], being probably the only region available in SP-C to sustain interactions with other bilayers or monolayers in surfactant. Numerous studies have characterized the lipid–protein interactions and lipid-perturbing effects of SP-C, in an attempt to understand in molecular terms the ability of SP-C to modulate the surface-active properties of surfactant [24–27]. Palmitate chains as well as the positive charges in the N-terminal region have been found in some studies to be essential determinants for the surface active properties of SP-C [27–31], although other studies have proposed that the presence of the palmitate chains is not strictly required for the main biophysical features of SP-C [15]. A major problem is that most physicochemical features reported for SP-C are dominated by contributions from the hydrophobic α-helix, masking potentially relevant properties of the N-terminal segment of the protein. The occurrence of selective lipid–protein interactions involving this region of SP-C has been studied previously using protein forms that were chemically derivatized to introduce extrinsic probes at the N-terminal end [23,32]. The main goal of the present work was to detect and characterize potential interactions of a synthetic peptide designed from the N-terminal segment of SP-C with model phospholipid monolayers. We recently demonstrated that this peptide associates spontaneously with phospholipid membranes [33]. In the present work, we have also identified an intrinsic propensity of the N-terminal region of the protein to insert into and perturb phospholipid monolayers, even in the absence of the palmitoylation that is present in the N-terminal segment of the native protein. Such intrinsic lipid-perturbing properties are likely relevant to the participation of SP-C in pulmonary surfactant function.

2. Materials and methods

2.1. Materials

Chloroform (Chl) and methanol (MetOH) were HPLC-grade solvents from Scharlau (Barcelona, Spain). The lipids, dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG), and 1-acyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine (NBD-PC), were all purchased from Avanti Polar Lipids (Birmingham, AL, USA).

2.2. Peptide synthesis and purification

The peptide studied in this work was synthesized by Fmoc chemistry, purified by HPLC and analyzed by mass spectrometry, as previously described [33,34]. The 13-residue peptide pSP-C_{L1W}b, with sequence NH_2W-R-I-P-O-O-P-V-N-L-K-R-LCONH_2, was designed taking as a model the sequence of porcine SP-C. The N-terminal Leu residue in the native sequence was replaced in the synthetic peptide by Trp, with the aim of using it as intrinsic probe to characterize structure and lipid–protein interactions by fluorescence spectroscopy [34]. The two Cys residues in peptide pSP-C_{L1W}b have their side chains blocked by acetamidomethyl groups (O) to avoid potential peptide oligomerization. HPLC and mass analyses confirmed that more than 80% of the peptide maintained the two cysteines blocked, while a minor proportion had one blocked and one free cysteine, and that at least 95% of the peptide certainly conserved its monomeric character.

2.3. Peptide adsorption at the air–water interface

To follow adsorption of the peptide into an air–water interface, the concomitant increase in surface pressure ($\Delta\pi$) was monitorized with time (π–t isotherms) after injection of an small volume of methanolic peptide solution into the subphase. Injections of equivalent volumes of pure methanol did not produce any detectable change in surface pressure. Measurements were made in a Teflon trough similar to that described by King and Clements [35], designed for our laboratory by Nima, (Coventry, UK). The subphase consisted of 1.5 mL of 5 mM Tris buffer pH 7, containing 150 mM NaCl, thermostated at 25 °C and subjected to continuous stirring.

2.4. Interaction of the peptide with preformed monolayers

Changes in surface pressure ($\Delta\pi$) were monitored upon injection of small volumes of a methanolic peptide solution

underneath phospholipid monolayers preformed in the surface balance at different initial pressures π_i. Association with and eventual insertion of the peptide into the interfacial phospholipid film was detected by a subsequent increase in surface pressure. The critical insertion pressure (π_c) was defined as the maximum initial pressure π_i of the monolayer permitting peptide insertion, estimated from a plot of $\Delta\pi$ versus π_i.

Measurements were made under similar conditions to those described above. Different monolayers were first formed by spreading small volumes of a DPPC or DPPG solution (1 mg/mL) in Chl/MetOH (3:1, v/v), on top of the buffered subphase until the required pressure was achieved. After 15 min equilibration to allow for solvent evaporation, a small aliquot, typically 5–10 μL, of a concentrated peptide solution in methanol was injected into the subphase and the change in surface pressure was recorded.

2.5. π–A isotherms of lipid and lipid/peptide monolayers

The effect of the peptide on the compression isotherms of phospholipids monolayers was analyzed in a Langmuir–Wilhelmy surface balance (Applied Imaging, Dukesway Team Valley, Gateshead, UK), equipped with a Teflon through of 150 mL, thermostated at 22 ± 1 °C. Lipid/peptide monolayers were formed by first mixing the peptide and the phospholipid, DPPC or the mixture DPPC/DPPG (7:3, mol/mol) in Chl/MetOH (3:1, v/v) in the desired proportions. Lipid or lipid/peptide organic solution was then spread on top of a buffered (5 mM Tris, pH 7) subphase, containing 150 mM NaCl. Alternatively, monolayers of the same lipid and lipid/peptide mixtures were tested on a Teflon ribbon-barrier surface balance [36], which prevents leakage of monolayer components at high π and on a balance equipped with an epifluorescence microscopic attachment [37]. The monolayers initially occupied 160 cm^2 and were compressed at 20 mm^2/s in all the balances, surface pressure–area (π–A) data being collected using a Wilhelmy dipping plate attached to a force transducer [37]. In the epifluorescence microscopy experiments, the monolayers were compressed in 20 steps and a pause of 5 min was introduced at each step to monitor and record the visual features of the monolayer as described previously [37]. To observe the monolayer by fluorescence microscopy, 1% NBD-PC was also included in the phospholipid or phosholipid/peptide mixture prior to monolayer formation. The acquired fluorescence images were analyzed digitally using the program SigmaScan Pro 5 (©SPSS Inc).

2.6. Stability of compressed lipid and lipid/peptide monolayers

The effect of peptide on the stability of monolayers compressed to very high pressures was analyzed by compressing DPPC or DPPG monolayers with or without the peptide, up to 70 mN/m, under the same conditions explained above. Once this pressure was reached, compression was stopped and the spontaneous decline of surface pressure was monitored over time.

2.7. Statistics

Unless otherwise indicated, results have been presented as representative experiments after repeated examination of three different samples from at least two different batches of peptide. Epifluorescence images shown in the Figures are representative micrographs obtained after analysing three or more different films from either lipid or lipid/peptide monolayers, compressed to the indicated pressures. Quantitative data from epifluorescence images are shown as averaged values with standard deviations obtained after analysis of at least 5 different images at each selected surface pressure.

3. Results

3.1. Interaction with preformed monolayers

Preliminary studies showed that peptide pSP-C_{L1W}b could form by itself interfacial films when a small volume of peptide organic solution was spread directly on top of an aqueous surface [38]. We have now tested for peptide adsorption from the subphase into the interface to spontaneously form peptide films. Fig. 1 shows how the injection of a very small amount of peptide into the aqueous subphase promotes a practically instantaneous increase in surface pressure measured at the interface, as a consequence of the formation of peptide films. This effect confirms the amphipathic character of the peptide, leading to its stable incorporation at the air/liquid interface. Peptide concentrations higher than 0.57 μM saturated the interface, and no further increase of surface pressure was detected when higher amounts of peptide were injected. The maximum surface pressure measured in adsorbed peptide monolayers was in the range of 18–20 mN/m.

Interfacial adsorption of the peptide towards an interface already occupied by a DPPC or DPPG monolayer was assayed at different initial pressures, π_i. Fig. 2 illustrates that injection of the peptide in the subphase underneath a DPPC monolayer at 5 mN/m promotes an instantaneous pressure increase of 20 mN/m, as a consequence of the insertion of the peptide into the interface. A similar injection of peptide promoted very small effects, however, on the surface pressure of DPPC monolayers preformed at around 25 mN/m. When the peptide was injected underneath DPPG monolayers the peptide could insert into DPPG monolayers preformed at higher surface pressures than the maximal pressures permitting the insertion of pSP-C_{L1W}b in DPPC films. These differences suggest that lipid/peptide electrostatic interactions contribute significantly to the association and stabilization of the peptide with the interfacial films.

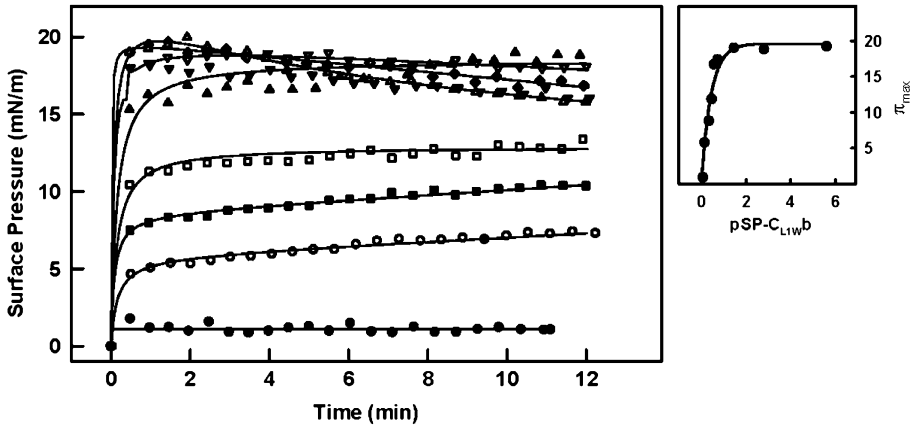

Fig. 1. Interfacial adsorption kinetics of peptide pSP-C$_{L1W}$b. π–t adsorption kinetics isotherms of different amounts of peptide pSP-C$_{L1W}$b injected at $t=0$ in a subphase composed of 5mM Tris buffer, pH 7, 150 mM NaCl, thermostated at 25 °C. Peptide concentration in the subphase was 0.08 (●), 0.15 (○), 0.34 (■), 0.46 (□), 0.57 (▲), 0.73 (△), 1.46 (▼), 2.8 (▽), and 5.6 (♦) μM. The right hand panel shows π_{max} versus peptide concentration (μM) in the subphase.

The critical pressure (π_c) for the insertion of the peptide in monolayers made of DPPC or DPPG has been estimated by representing the increase in pressure induced by the peptide as a function of the initial pressure of the monolayer (π_i) (Fig. 3). π_c represents the maximum π_i permitting insertion of the peptide into the preformed monolayers, as detected by a subsequent increase in surface pressure. The comparison of π_c for monolayers with different composition provides information about the nature and extent of the peptide–lipid interactions at the interface. It is generally assumed that the lateral packing in a lipid bilayer can be roughly mimicked by a monolayer compressed to π around 30–35 mN/m. Molecules showing insertion π_c higher than 30 mN/m are then considered as potentially competent to interact and insert into lipid membranes [39]. The π_c calculated for the insertion of pSP-C$_{L1W}$b in DPPC monolayers was around 37 mN/m, while the π_c for insertion in preformed DPPG films was estimated to be as high as 55 mN/m. This result was consistent with the behavior observed previously for interaction of the peptide with bilayers, as pSP-C$_{L1W}$b interacted with higher affinity and to a greater extent with DPPG than with DPPC membranes. This differential behavior was interpreted as a consequence of the participation of electrostatic lipid–peptide interactions in inducing in the peptide a conformation which enable it to penetrate deeper into the interface in anionic membranes [33].

3.2. Organization of lipid and lipid/peptide films

The effect of the interaction of the peptide on the lipid packing in the monolayers was analyzed by epifluorescence microscopy. Fig. 4 shows the π–A isotherm of a DPPC film in the absence and in the presence of 10% or 20% peptide pSP-C$_{L1W}$b by weight (aprox. 4 mol% and 8 mol% peptide in the peptide–lipid system, respectively). The peptide expanded the isotherms, interpreted as resulting from the area occupied by the peptide molecules themselves plus the peptide-dependent expansion of the intrinsic area/molecule of the lipid. The presence of the peptide also broadened and raised the surface pressure of the liquid-expanded to liquid condensed transition plateau that occurred in DPPC at around 11 mN/m [40]. The lipid and lipid/peptide isotherms converged at around 28 mN/m, indicating that the peptide is

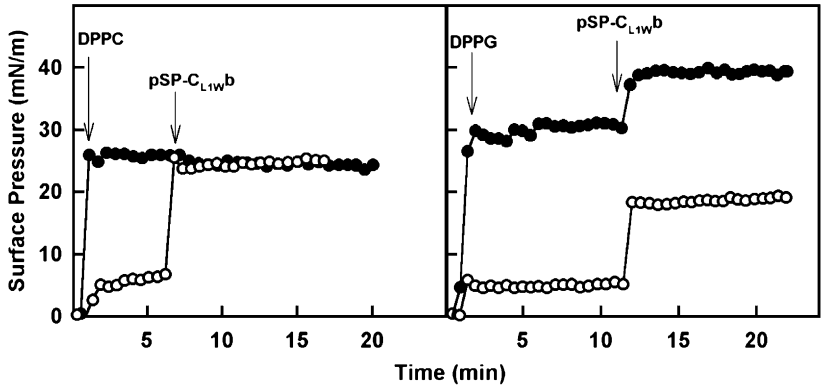

Fig. 2. Insertion kinetics of peptide pSP-C$_{L1W}$b into preformed phospholipid monolayers. Insertion kinetics of pSP-C$_{L1W}$b 2.8 μM in DPPC (left) or DPPG (right) monolayers preformed at different pressures. Initial pressure (π_i) was of 5 (○) or 27 (●) mN/m, and the through was maintained at 25±1 °C. The subphase was composed of 5 mM Tris buffer, pH 7, containing 150 mM NaCl.

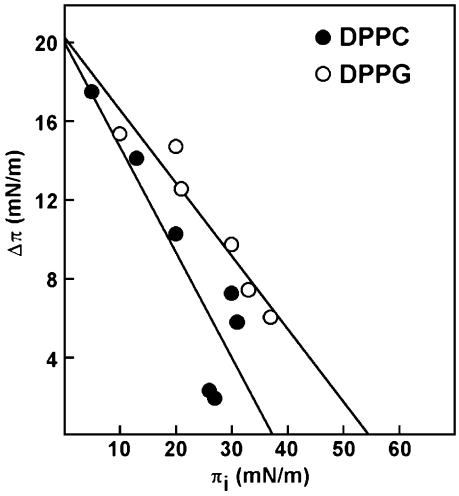

Fig. 3. Determination of the critical pressure for the insertion of peptide pSP-C_{L1W}b into preformed monolayers. Increase in surface pressure ($\Delta\pi$) vs. initial pressure (π_i) of preformed DPPC (●) or DPPG (○) monolayers upon injection of peptide pSP-C_{L1W}b. The subphase was composed of a buffer of 5 mM Tris , pH 7, containing 150 mM NaCl, maintained at 25 °C. The peptide concentration in all the experiments was 2.8 μM. The lines represent the linear regression that best fits the experimental data. The intersection with the abscissa estimates the critical insertion pressure (π_c) for each type of film.

squeezed-out of the interface at pressures higher than this value. This squeeze-out or exclusion pressure, π_e, is below the π_c estimated for the insertion of the peptide. π_e is probably dependent on both packing and the compression rate, whereas π_c is dominated by packing density. If the isotherms would be obtained using slow enough compression rates, both π_c and π_e would probably be more similar. The slight displacement of the high pressure-region of the lipid/peptide isotherm to smaller areas per phospholipid molecule compared with the isotherm of pure DPPC suggests that the peptide may be carrying some lipids out of the film when it is being squeezed-out from the interface (or the intrinsic packing density of the lipid is changing). Fig. 4 shows epifluorescence images taken at different pressures during compression of DPPC or DPPC/ pSP-C_{L1W}b films doped with 1 mol% of the fluorescent lipid probe NBD-PC. Compression of pure DPPC films induced the appearance of a typical liquid-expanded-to-liquid-condensed two-dimensional transition, including nucleation and growth of dark kidney been-shaped probe-depleted condensed domains up to a coverage of more than 60% of the total area at pressures above 15 mN/m. Fig. 5 provides a quantitative description of the influence of the peptide on the distribution and characteristics of the fluid and condensed in DPPC monolayers. Presence of the peptide caused a very significant reduction of the area occupied by the liquid-condensed phase, which can be interpreted as a consequence of peptide-induced inhibition of the nucleation of condensed domains [24,41]. The peptide also modified the morphology of the film during the phase transition, which occurred in the form of much more numerous but smaller domains. This suggests that peptide pSP-C_{L1W}b penetrates into the hydrophobic region of the interfacial monolayer, perturbing the packing of the lipid chains and preventing their participation in the compression-driven lateral transition.

Fig. 6 illustrates the effect of peptide pSP-C_{L1W}b on DPPC/DPPG (7:3, mol/mol) monolayers. The effect of peptide on the π–A isotherm of this type of lipid film is qualitatively similar to that observed in monolayers composed exclusively of DPPC. The peptide caused a similar expansion of the isotherm and was squeezed-out from the interface, although at somewhat higher pressures, around 33 mN/m, than it was from purely zwitterionic films. The latter observation indicates that electrostatic lipid/peptide interactions contribute to the stabilization and retention of the peptide at the interface at higher pressures. Fig. 7 provides a quantitative analysis of the influence of the peptide on DPPC/DPPG films. Interestingly, the effect of the peptide on the inhibition of the condensation of anionic films was less pronounced than it was in DPPC monolayers (see Fig. 7 and compare with Fig. 5). The total amount of phase condensed by compression of DPPC/DPPG films above 10 mN/m was only slightly smaller in the presence of the peptide than in its absence. However, as occurred in DPPC/ pSP-C_{L1W}b films, the condensed phase in DPPC/DPPG pSP-C_{L1W}b layers was distributed in much more numerous but smaller domains than those formed in the absence of the peptide. This probably indicates that the N-terminal SP-C peptide penetrates into the hydrophobic interfacial layer of DPPC/DPPG films to similar extents as it does into those composed of pure DPPC, therefore similarly inhibiting the nucleation of condensed domains, which therefore appear at higher pressures and at more numerous points of the films. The higher condensation of DPPC/DPPG/ pSP-C_{L1W}b films compared with those made of DPPC/ pSP-C_{L1W}b probably has to do with the effect of the electrostatic lipid/peptide interactions established by the peptide acting as a polycation. Charge neutralization in the peptide-DPPC/DPPG films mayhave allowed for greater packing density and total condensed phase than that in the peptide-DPPC films. The peptide pSP-C_{L1W}b would apparently have, therefore, contradictory effects. It would inhibit nucleation of solid domains via perturbation of chain packing coupled with penetration of the peptide into the acyl chain regions, but, once domains nucleate overcoming the peptide-disordering effect, the polycationic nature of pSP-C_{L1W}b would contribute to condensation of more lipid molecules during the growing of the domains.

3.3. Stability of monolayer compressed states

To evaluate the effect of the presence of the N-terminal SP-C peptide on the stability of phospholipid/peptide films compressed to high pressures we have monitored the spontaneous relaxation occurring in DPPC or DPPG films compressed to 70 mN/m, in the absence or in the presence of 20% by weight of peptide pSP-C_{L1W}b (Fig. 8). Both

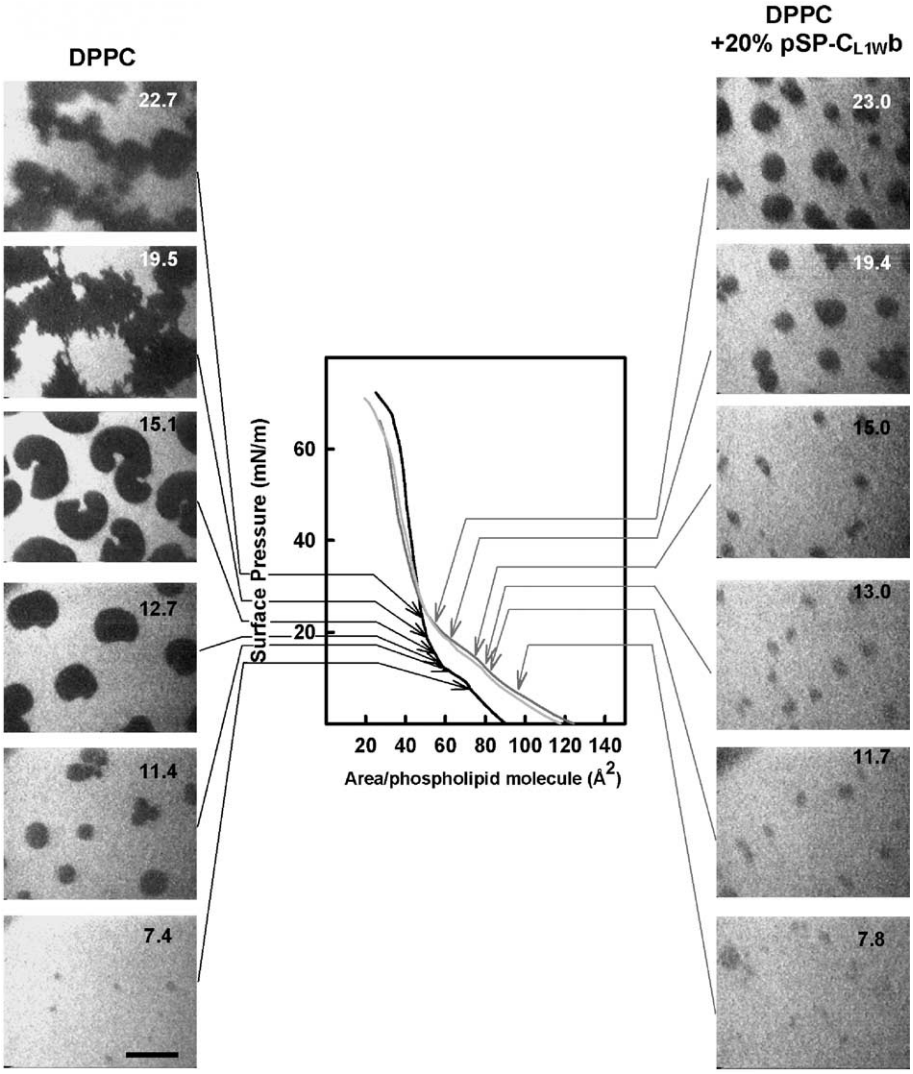

Fig. 4. Effect of peptide pSP-C_{L1W}b on the π–A isotherm and the organization of DPPC monolayers. π–A compression isotherm of a DPPC monolayer in the absence (black line) or in the presence of 10% (light grey) or 20% (dark grey) of peptide pSP-C_{L1W}b by weight (4 and 8 mol%, respectively). Images are epifluorescence microscopy pictures taken from films of pure DPPC (left frames) or DPPC/pSP-C_{L1W}b (right frames) compressed to the indicated pressures. The films contained NBD-PC (1% mol/mol) to allow visualization of the phospholipid distribution at the interface. The surface balance was maintained at 22±1 °C. The scale bar represents 100 μm.

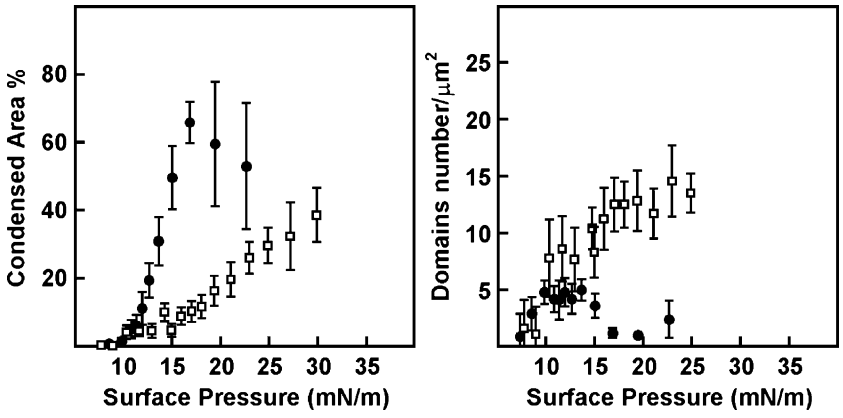

Fig. 5. Effect of peptide pSP-C_{L1W}b on the compression-driven condensation of DPPC films. Quantitative analysis of the total proportion of condensed area (left panel) and the density of condensed domains (right panel) observed by epifluorescence microscopy in DPPC monolayers in the absence (●) or in the presence (□) of 20% peptide pSP-C_{L1W}b (weight/weight), as a function of the surface pressure. Data plotted represent means±S.D. after averaging 5 images at each pressure.

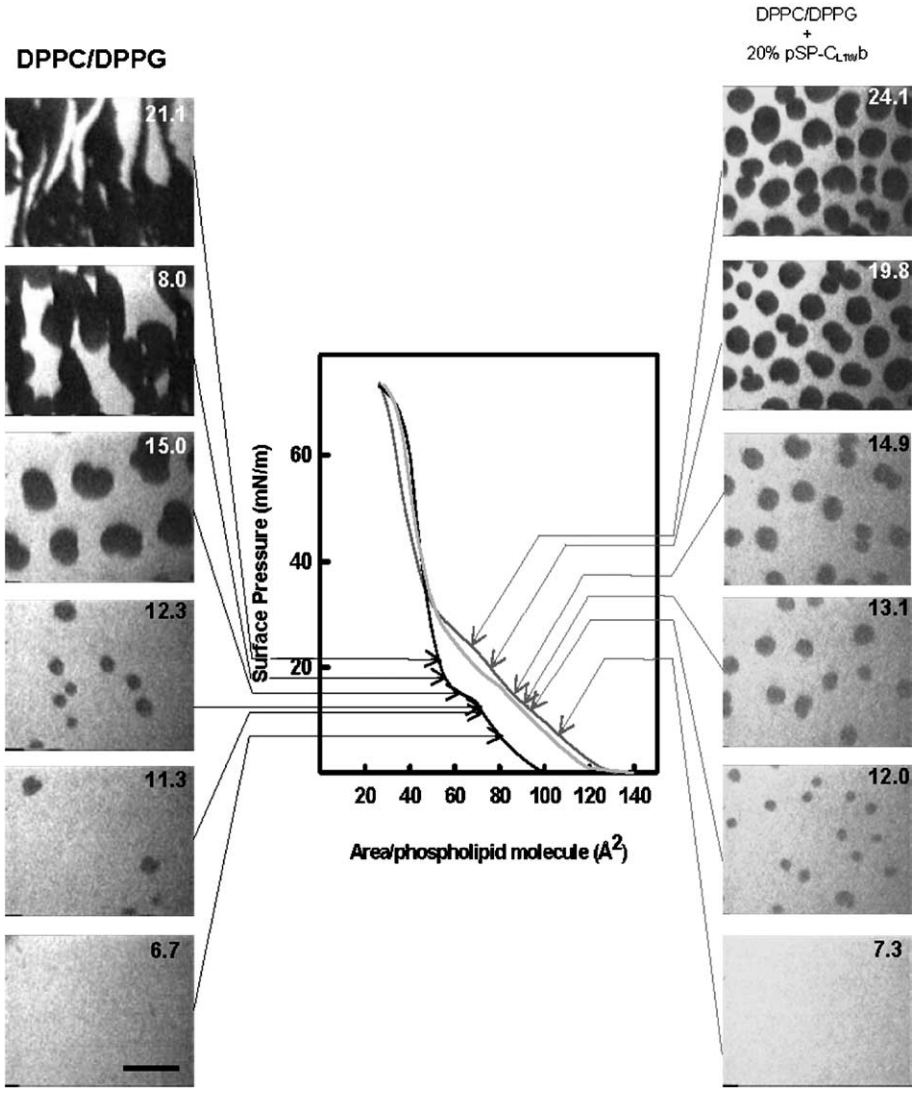

Fig. 6. Effect of peptide pSP-C_{L1W}b on the π–A isotherm and the organization of DPPC/DPPG (7:3, mol/mol) monolayers. π–A compression isotherms of a DPPC/DPPG monolayer in the absence (black line) or in the presence of 10% (light grey) or 20% (dark grey) of peptide pSP-C_{L1W}b by weight (4 and 8 mol%, respectively). Images are epifluorescence microscopy pictures taken form DPPC/DPPG (left frames) or DPPC/DPPG/pSP-C_{L1W}b (right frames) films compressed to the indicated pressures. The films contained NBD-PC (1% mol/mol) to allow observation of the phospholipid distribution at the interface. The through was maintained at 22 ± 1 °C. Scale bar represents 100 μm.

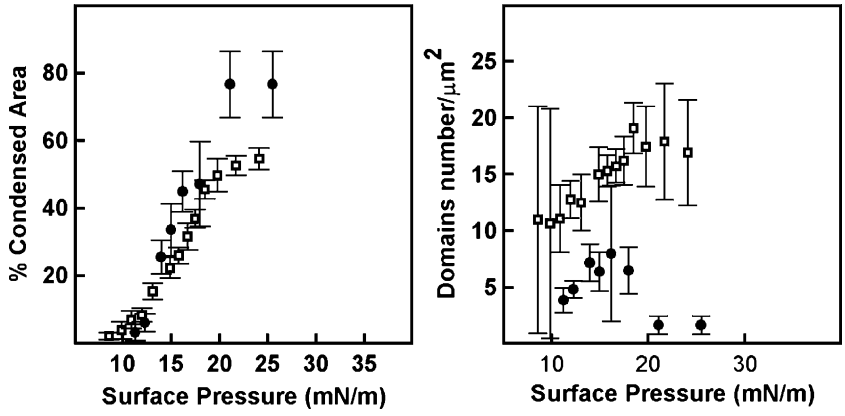

Fig. 7. Effect of peptide pSP-C_{L1W}b on the compression-driven condensation of DPPC/DPPG (7:3, mol/mol) films. Quantitative analysis of the total proportion of condensed area (left panel) and the density of condensed domains (right panel) observed by epifluorescence microscopy in DPPC/DPPG (7:3, mol/mol) monolayers in the absence (●) or in the presence (□) of 20% peptide pSP-C_{L1W}b (weight/weight), as a function of the surface pressure. Data plotted represent means ± S.D. after averaging 5 frames at each pressure.

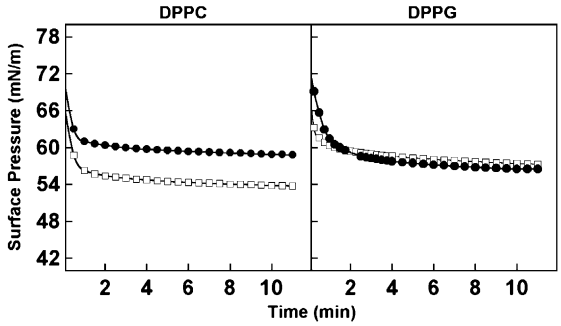

Fig. 8. Effect of peptide pSP-C_{L1W}b on the stability of compressed phospholipid monolayers. $\pi - t$ relaxation kinetics of DPPC (left panel) or DPPG (right panel) monolayers in the absence (●) or in the presence (□) of 20% by weight peptide pSP-C_{L1W}b, after compression to 70 mN/m. The monolayers were prepared by spreading a phospholipid or phospholipid/peptide organic solution on top of a subphase composed of 5 mM Tris buffer, pH 7, containing 150 mM NaCl held at 22±1 °C.

DPPC and DPPG monolayers can be compressed to over 70 mN/m when they are at temperatures below the main gel-to-liquid crystalline phase transition of their comparable bilayers (around 41 °C for both phospholipids). This compressed state is metastable and the films relax slowly from that pressure to the equilibrium surface pressure, around 48 mN/m. Under the conditions of our experiments, including the particular geometry of our balance and a relatively rapid compression rate, the π–t isotherms obtained show biphasic relaxation kinetics, including a rapid fall in pressure to around 55–60 mN/m followed by a much slower and progressive decay to the equilibrium values.

Peptide pSP-C_{L1W}b increased the relaxation of the DPPC monolayer, an effect mainly occurring during the first rapid step. This destabilizing effect of the peptide on DPPC films was dependent on the proportion of peptide present in the monolayers (data not shown). The perturbation induced by the peptide on the lipid packing of the films is probably facilitating a faster ejection of lipid molecules from the highly compressed films after compression is stopped. Interestingly, the peptide pSP-C_{L1W}b did not cause significant effects on the relaxation kinetics of DPPG films (Fig. 8, right panel) suggesting again that electrostatic lipid–peptide interactions can at least partially compensate for destabilization due to peptide penetration into the interfacial films.

4. Discussion

The current model for the structure and disposition of SP-C in surfactant phospholipid bilayers and monolayers implicitly assumes that the N-terminal segment of the protein is associated with the membranes via the palmitoylated cysteines [16,42]. The results shown here demonstrate that the amino acid sequence of the N-terminal sequence of SP-C has an intrinsic propensity to spontaneously interact and associate with phospholipid interfacial monolayers. We have previously reported a similar propensity of the peptide to interact with phospholipid bilayers, and proposed that it is a consequence of the conformation of this segment, potentially an amphipathic β-hairpin [33].

The N-terminal segment is able to interact with both zwiterionic and anionic phospholipid monolayers, although the affinity and extent of the lipid–peptide interaction is significantly higher with negatively-charged layers, indicating the important contribution of electrostatic interactions with this cationic region of SP-C. The electrostatic component of the SP-C lipid–protein interaction have been analyzed in different studies [26,43], and we have previously determined that the N-terminal segment adopts a different disposition and/or conformation in the presence of anionic phospholipids [23]. The presence of positively charged residues in the SP-C N-terminal segment at physiological *p*H seems to be essential for the biophysical activities of the protein [28] also suggesting that specific interactions between the N-terminal segment and anionic phospholipids are of functional significance.

Mammalian pulmonary surfactant contains approximately 8% anionic phospholipids [44], mainly PG and phosphatidylinositol (PI). The negative charge provided to the monolayer by these lipid species probably mediates the final conformation and disposition of the SP-C N-terminal segment as it influences the insertion of SP-C into DPPG monolayers. Formation of selective SP-C/PG complexes in native surfactant has been proposed to underlie the mechanisms of some essential processes in surfactant dynamics such as sorting and squeeze-out of poorly surface active species from the interface during compression [45–47]. This model is based on the fact that squeeze-out of full length native SP-C from compressed protein–lipid films formed directly at the interface is accompanied of squeeze out of a significant amount of associated lipids. The present experiments indicate that the N-terminal segment of SP-C could stabilize SP-C–lipid interaction promoting resistance to exclusion, whereas the hydrophobic moiety of SP-C would help to carry out lipids out of the monolayer when the protein is excluded during compression.

A feature with important implications is the ability of the SP-C N-terminal segment to perturb to some extent lipid packing in bilayers and monolayers. This perturbation can be also observed macroscopically through the capacity of this peptide to induce aggregation and leakage of lipid vesicles [33]. Insertion into and perturbation by this protein motif of the structure of the phospholipid films could be part of the molecular mechanism enabling SP-C to catalyze lipid transfer from membranes to the interface [13,14,48] or forming and sustaining surface-associated surfactant reservoirs [14]. Li et al. [49] have recently shown that a pathological form of SP-C, bearing 12 extra residues from the SP-C precursor as an extension of the N-terminal segment of the protein, shows poorer ability to promote phospholipid adsorption, gives higher surface tension during cyclic film compression and has lower ability to

bind LPS, than native SP-C. It has been proposed that interaction of SP-C with LPS is also mediated by interactions at the N-terminal segment of the protein [50]. The results of Li and co-workers implicate the lipid–protein interactions of the N-terminal segment of the protein in critical activities of SP-C. At the physicochemical level, the perturbations introduced by the peptide would be the basis for this region of SP-C to modify the thermotropic [33] and barotropic phase transitions of bilayers and monolayers, respectively. Such alterations indicate that the N-terminal peptide affects relatively deep (hydrophobic) regions of the different lipid organizations, suggesting that, independently of the electrostatic component, the interaction of the N-terminal segment with membranes and monolayers has a significant hydrophobic contribution. The hydrophobic component of the interaction of SP-C with phospholipids has been studied [24,25,51] but it has been always assigned to the hydrophobic α-helix, encompassing residues 13–35 of the protein, these having a transmembrane disposition in membranes. The present results provide evidence that the N-terminal segment of SP-C also has a hydrophobic component in its interaction with monolayers, even in the absence of palmitoylated cysteines. The question is, then, what is the effect of the palmitoylation of cysteines on the peptide–monolayer interactions. A recent study showed that while the non-palmitoylated peptide was squeezed-out irreversibly from lipid/peptide films at relatively low pressures, a palmitoylated version maintained association with the interfacial films in highly compressed states, permitting an efficient and rapid re-insertion of the peptide into the interface upon expansion of the films [34]. Some of the perturbations caused by the SP-C N-terminal peptide in phospholipid films are shown in our experiments at relatively low pressures, below the pressures presumably existing at the interfacial films in vivo, at least in homeothermic mammals. The effects produced by this motif of SP-C on the lipid packing of compressed monolayers together with the simultaneous perturbations introduced on the structure of associated bilayers might be sufficient to promote or at least initiate transfer of phospholipids into the interface. One should not discard the possibility that physiological parameters such as the full compositional complexity of surfactant complexes, environmental temperatures around 37 °C, or the presence of calcium in the hypophase, might contribute to optimize the surface activity of SP-C at the particular conditions imposed by the alveolar spaces.

In some models of multiply-layered surface films of surfactant association between layers–bilayer to bilayer or bilayer to monolayer–is pictured as being influenced by SP-C with its hydrophobic helix in one layer and its palmitate(s) in another. This work suggests that even if both its palmitates and its helix were buried within the same layer, SP-C could still promote substantial interlayer contact through the intrinsic hydrophobic character of the N-terminal peptide itself, as well as, of course, through its intrinsic positive charge. This sort of SP-C promoted interaction between layers would allow for more facile and energetically favorable association and dissociation than if the two dominant parts of the SP-C were inserted into different layers.

We conclude, in summary, that the intrinsic structure defined by the amino acid sequence of the N-terminal segment of SP-C would be responsible for the ability of this motif to access the deep regions of the monolayer, which is presumably important in promoting lipid interfacial transfer, both during adsorption and during re-insertion upon expansion. Palmitoylation would be required to avoid loss of the protein–monolayer association at the end of expiration. The results of the present work are a further confirmation of the importance of the compression-dependent disposition and lipid–protein perturbing properties of the N-terminal segment of SP-C for the role of the whole protein in surfactant activities during the respiratory cycle.

Acknowledgements

We are grateful to Dr. Luis Rivas for technical assistance with the synthesis of the peptide and A. Cruz for his help with the epifluorescence image analysis. I. P. was recipient of a grant Severo Ochoa from Fundación Ferrer. This research has been supported by grants from Direccion General de Educacion Superior e Investigacion Cientifica (BIO2003-09056) and Comunidad de Madrid (08.2/0054.1/2001) to J. P.-G. and the Canadian Institutes of Health Research and Health Canada to K. M. W. K.

References

[1] C.B. Daniels, S. Orgeig, Pulmonary surfactant: the key to the evolution of air breathing, News Physiol. Sci. 18 (2003) 151–157.
[2] J. Goerke, Pulmonary surfactant: functions and molecular composition, Biochim. Biophys. Acta 1408 (1998) 79–89.
[3] J. Pérez-Gil, K.M.W. Keough, Interfacial properties of surfactant proteins, Biochim. Biophy. Acta 1408 (1998) 203–217.
[4] S. Schurch, R. Qanbar, H. Bachofen, F. Possmayer, The surface-associated surfactant reservoir in the alveolar lining, Biol. Neonate 67 (1995) 61–76.
[5] S. Schurch, H. Bachofen, F. Possmayer, Surface activity in situ, in vivo, and in the captive bubble surfactometer, Comp. Biochem. Physiol., Part A Mol. Integr. Physiol. 129 (2001) 195–207.
[6] J. Pérez-Gil, Lipid–protein interactions of hydrophobic proteins SP-B and SP-C in lung surfactant assembly and dynamics, Pediatr. Pathol. Mol. Med. 20 (2001) 445–469.
[7] J.A. Whitsett, T.E. Weaver, Hydrophobic surfactant proteins in lung function and disease, N. Engl. J. Med. 347 (2002) 2141–2148.
[8] S.W. Glasser, M.S. Burhans, T.R. Korfhagen, C.L. Na, P.D. Sly, G.F. Ross, M. Ikegami, J.A. Whitsett, Altered stability of pulmonary surfactant in SP-C-deficient mice, Proc. Natl. Acad. Sci. U. S. A. 8 (2001) 6366–6371.
[9] L.M. Nogee, A.E. Dunbar III, S.E. Wert, F. Askin, A. Hamvas, J.A. Whitsett, A mutation in the surfactant protein C gene associated with familial interstitial lung disease, N. Engl. J. Med. 344 (2001) 573–579.
[10] L.M. Nogee, Abnormal expression of surfactant protein C and lung disease, Am. J. Respir. Cell. Mol. Biol. 26 (2002) 641–644.

[11] L.M. Nogee, Alterations in SP-B and SP-C expression in neonatal lung disease, Annu. Rev. Physiol. 66 (2004) 601–623.
[12] E.J. Veldhuizen, H.P. Haagsman, Role of pulmonary surfactant components in surface film formation and dynamics, Biochim. Biophys. Acta 1467 (2000) 255–270.
[13] J. Pérez-Gil, J. Tucker, G. Simatos, K.M.W. Keough, Interfacial adsorption of simple lipid mixtures combined with hydrophobic surfactant protein from pig lung, Biochem. Cell. Biol. 70 (1992) 332–338.
[14] E.J. Veldhuizen, R.V. Diemel, G. Putz, L.M. van Golde, J.J. Batenburg, H.P. Haagsman, Effect of the hydrophobic surfactant proteins on the surface activity of spread films in the captive bubble surfactometer, Chem. Phys. Lipids 110 (2001) 47–55.
[15] F. Possmayer, K. Nag, K. Rodriguez, R. Qanbar, S. Schurch, Surface activity in vitro: role of surfactant proteins, Comp. Biochem. Physiol., Part A Mol. Integr. Physiol. 129 (2001) 209–220.
[16] J. Johansson, Structure and properties of surfactant protein C, Biochim. Biophys. Acta 1408 (1998) 161–172.
[17] J. Johansson, T. Szyperski, T. Curstedt, K. Wuthrich, The NMR structure of the pulmonary surfactant-associated polypeptide SP-C in an apolar solvent contains a valyl-rich alpha-helix, Biochemistry 33 (1994) 6015–6023.
[18] J. Johansson, T. Szyperski, K. Wuthrich, Pulmonary surfactant-associated polypeptide SP-C in lipid micelles: CD studies of intact SP-C and NMR secondary structure determination of depalmitoyl-SP-C(1–17), FEBS Lett. 362 (1995) 261–265.
[19] T. Curstedt, J. Johansson, P. Persson, A. Eklund, B. Robertson, B. Löwenadler, H. Jörnvall, Hydrophobic surfactant-associated polypeptides: SP-C is a lipopeptide with two palmitoylated cysteine residues, whereas SP-B lacks covalently linked fatty aacyl groups, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 2985–2989.
[20] 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 (1991) 10058–10064.
[21] G. Vandenbussche, A. Clercx, T. Curstedt, J. Johansson, H. Jornvall, J.M. Ruysschaert, Structure and orientation of the surfactant associated protein C in a lipid bilayer, Eur. J. Biochem. 203 (1992) 201–209.
[22] M.R. Morrow, S. Taneva, G.A. Simatos, L.A. Allwood, K.M. Keough, 2H NMR studies of the effect of pulmonary surfactant SP-C on the 1,2-dipalmitoyl-sn-glycero-3-phosphocholine headgroup: a model for transbilayer peptides in surfactant and biological membranes, Biochemistry 32 (1993) 11338–11344.
[23] I. Plasencia, A. Cruz, C. Casals, J. Perez-Gil, Superficial disposition of the N-terminal region of the surfactant protein SP-C and the absence of specific SP-B–SP-C interactions in phospholipid bilayers, Biochem. J. 359 (2001) 651–659.
[24] J. Pérez-Gil, K. Nag, S. Taneva, K.M.W. Keough, Pulmonary surfactant protein SP-C causes packing rearrangements of dipalmitoylphosphatidylcholine in spread monolayers, Biophys. J. 63 (1992) 197–204.
[25] K. Shiffer, S. Hawgood, H.P. Haagsman, B. Benson, J.A. Clements, J. Goerke, Lung surfactant proteins, SP-B and SP-C, alter the thermodynamic properties of phospholipid membranes: a differential calorimetry study, Biochemistry 32 (1993) 590–597.
[26] J. Pérez-Gil, C. Casals, D. Marsh, Interactions of hydrophobic lung surfactant proteins SP-B and SP-C with dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylglicerol bilayers studied by electron spin resonance spectroscopy, Biochemistry 34 (1995) 3964–3971.
[27] Z. Wang, O. Gurel, J.E. Baatz, R.H. Notter, Acylation of pulmonary surfactant protein-C is required for its optimal surface active interactions with phospholipids, J. Biol. Chem. 271 (1996) 19104–19109.
[28] L.A.J.M.B. Creuwels, E.H. Demel, R.A. Vangolde, L.M.G., H.P. Haagsman, Neutralization of the positive charges of surfactant protein C—effects on structure and function, J. Biol. Chem. 270 (1995) 16225–16229.
[29] L.A.J.M. Creuwels, R.A. Demel, L.M.G. VanGolde, H.P. Haagsman, Characterization of a dimeric canine form of surfactant protein C (SP-C), Biochim. Biophys. Acta 1254 (1995) 326–332.
[30] C.R. Flach, A. Gericke, K.M.W. Keough, R. Mendelsohn, Palmitoylation of lung surfactant protein SP-C alters surface thermodynamics, but not protein secondary structure or orientation in 1,2-dipalmitoylphosphatidylcholine Langmuir films, Biochim. Biophy. Acta 1416 (1999) 11–20.
[31] L.A.J. Creuwels, R.A. Demel, L.M.G. VanGolde, B.J. Benson, H.P. Haagsman, Effect of acylation on structure and function of surfactant protein C at the air–liquid interface, J. Biol. Chem. 268 (1993) 26752–26758.
[32] I. Plasencia, A. Cruz, J.L. Lopez-Lacomba, C. Casals, J. Perez-Gil, Selective labeling of pulmonary surfactant protein SP-C in organic solution, Anal. Biochem. 296 (2001) 49–56.
[33] I. Plasencia, L. Rivas, K.M. Keough, D. Marsh, J. Perez-Gil, The N-terminal segment of pulmonary surfactant lipopeptide SP-C has intrinsic propensity to interact with and perturb phospholipid bilayers, Biochem. J. 377 (2004) 183–193.
[34] X. Bi, C.R. Flach, J. Perez-Gil, I. Plasencia, D. Andreu, E. Oliveira, R. Mendelsohn, Secondary structure and lipid interactions of the N-terminal segment of pulmonary surfactant SP-C in Langmuir films: IR reflection-absorption spectroscopy and surface pressure studies, Biochemistry 41 (2002) 8385–8395.
[35] R.J. King, J.A. Clements, Surface active materials from dog lung: II. Composition and physiological correlations, Am. J. Physiol. 223 (1972) 715–726.
[36] S.G. Taneva, K.M.W. Keough, Dynamic surface properties of pulmonary surfactant proteins SP-B and SP-C and their mixtures with dipalmitoylphosphatidylcholine, Biochemistry 33 (1994) 14660–14670.
[37] K. Nag, K. Boland, N. Rich, K.M.W. Keough, Design and construction of an epifluorescence microscopic surface balance for the study of lipid monolayer phase transitions, Rev. Sci. Instrum. 61 (1990) 3425–3430.
[38] I. Plasencia, L. Rivas, C. Casals, K.M. Keough, J. Perez-Gil, Intrinsic structural differences in the N-terminal segment of pulmonary surfactant protein SP-C from different species, Comp. Biochem. Physiol. A Mol. Integr. Physiol. 129 (2001) 129–139.
[39] H. Brockman, Lipid monolayers: why use half a membrane to characterize protein–membrane interactions? Curr. Opin. Struct. Biol. 9 (1999) 438–443.
[40] A. Cruz, L. Vazquez, M. Velez, J. Perez-Gil, Effect of pulmonary surfactant protein SP-B on the micro- and nanostructure of phospholipid films, Biophys. J. 86 (2004) 308–320.
[41] K. Nag, S.G. Taneva, J. Perez-Gil, A. Cruz, K.M. Keough, Combinations of fluorescently labeled pulmonary surfactant proteins SP-B and SP-C in phospholipid films, Biophys. J. 72 (1997) 2638–2650.
[42] A. ten Brinke, J.J. Batenburg, B.M. Gadella, H.P. Haagsman, A.B. Vaandrager, L.M. van Golde, The juxtamembrane lysine and arginine residues of surfactant protein C precursor influence palmitoylation via effects on trafficking, Am. J. Respir. Cell. Mol. Biol. 25 (2001) 156–163.
[43] M. Palmblad, J. Johansson, B. Robertson, T. Curstedt, Biophysical activity of an artificial surfactant containing an analogue of surfactant protein (SP)-C and native SP-B, Biochem. J. 339 (1999) 381–386.
[44] S. Hawgood, in: R.G. Crystal, J.B. West, P.J. Barnes, E.R. y Weibel (Eds.), The Lung: Scientific Foundations, Lippincott-Raven, Philadelphia, 1997, pp. 557–571.
[45] S. Taneva, K.M. Keough, Pulmonary surfactant proteins SP-B and SP-C in spread monolayers at the air–water interface: III. Proteins SP-B plus SP-C with phospholipids in spread monolayers, Biophys. J. 66 (1994) 1158–1166.
[46] K. Rodriguez-Capote, K. Nag, S. Schurch, F. Possmayer, Surfactant protein interactions with neutral and acidic phospholipid films, Am. J. Physiol., Lung Cell. Mol. Physiol. 281 (2001) L231–L242.
[47] B. Pastrana-Rios, C.R. Flach, J.W. Brauner, A.J. Mautone, R. Mendelsohn, A direct test of the “squeeze-out” hypothesis of lung

surfactant function. External reflection FT-IR at the air/water interface, Biochemistry 33 (1994) 5121–5127.
[48] M.A. Oosterlaken-Dijksterhuis, H.P. Haagsman, L.M.G. van Golde, R.A. Demel, Interaction of lipid vesicles with monomolecular layers containing lung surfactant proteins SP-B or SP-C, Biochemistry 30 (1991) 8276–8281.
[49] J. Li, M. Ikegami, C.L. Na, A. Hamvas, Q. Espinassous, R. Chaby, L.M. Nogee, T.E. Weaver, J. Johansson, N-terminally extended surfactant protein (SP) C isolated from SP-B-deficient children has reduced surface activity and inhibited lipopolysaccharide binding, Biochemistry 43 (2004) 3891–3898.
[50] L.A. Augusto, J. Li, M. Synguelakis, J. Johansson, R. Chaby, Structural basis for interactions between lung surfactant protein C and bacterial lipopolysaccharide, J. Biol. Chem. 277 (2002) 23484–23492.
[51] G.A. Simatos, K.B. Forward, M.R. Morrow, K.M.W. Keough, Interaction between perdeuterated dimyristoylphosphatidylcholine and low molecular weight pulmonary surfactant protein SP-C, Biochemistry 29 (1990) 5807–58114.