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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

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Abstract

Surfactant protein C (SP-C) is synthesized in the alveolar type II cells of the lung as a 21 kDa propeptide which is proteolytically processed to a 4.2 kDa mature active form. The main function of this extremely hydrophobic protein is to enhance lipid insertion into the air/liquid interface in the lung upon inhalation. This is necessary to maintain a relatively low surface tension at this interface during breathing. In this report we describe the production of mature human SP-C in the baculovirus expression system. The recombinant protein contains a secondary structure with a high α-helical content (73%), comparable to native SP-C, as determined by circular dichroism and attenuated total reflection Fourier transform infrared analysis. The expressed protein is a mixture of dipalmitoylated (15%) and non-palmitoylated SP-C. This suggests that the information required for palmitoylation is contained within the sequence of the mature protein. The activity of the protein to insert phospholipids into a preformed monolayer of lipids at an air/liquid interface was determined with a captive bubble surfactometer. Recombinant SP-C significantly reduced the surface tension at the air/liquid interface during dynamic expansion and compression. We conclude that correctly folded, dipalmitoylated and active SP-C can be expressed in the baculovirus expression system. Our results may facilitate investigations into the relation between structure and function of SP-C and into protein palmitoylation in general.

Keywords: Pulmonary surfactant protein C; Palmitoylation; Baculovirus; Captive bubble surfactometer; (Lung)

Abbreviations: hSP-C, human surfactant protein C; rSP-C, recombinant surfactant protein C; pSP-C, porcine surfactant protein C; ata, absolute atmospheric pressure; γ, surface tension; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol); DCM, dichloromethane; ACN, acetonitrile; TFA, trifluoroacetic acid; ATR-FTIR, attenuated total reflection-Fourier transform infrared; ESI, electrospray ionization; HPLC, high performance liquid chromatography; SUV, small unilamellar vesicles; ELSD, evaporative light scattering detector; CBS, captive bubble surfactometer

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1. Introduction

Surfactant protein C (SP-C) is a component of pulmonary surfactant. This mixture of lipids and proteins is secreted by the alveolar type II cells. Its main function is to reduce the surface tension at the air/liquid interface of the lung by forming a surface active film enriched in DPPC. This facilitates breathing and prevents the alveoli from collapsing at low lung volumes. SP-C (as well as surfactant protein B) has been demonstrated to enhance lipid insertion into the surface film at the air/liquid interface. A rapid insertion of lipids is needed to maintain a relatively low surface tension upon inhalation (for reviews, see [1,2]).

Within the lung, SP-C is synthesized as a 21 kDa precursor protein that is processed to yield a 4.2 kDa mature form. Both N-terminal and C-terminal precursor parts are cleaved off en route to or within the lamellar bodies of the type II cells [3,4]. The function of these precursor parts is not yet fully understood, but a 22 amino acid region in the C-terminus is thought to be required for post-translational targeting of the protein [5,6].

The mature protein is extremely hydrophobic and well conserved among species [7]. It consists of a hydrophobic stretch, rich in valines, which adopts an α-helix that can span a lipid bilayer [8]. Adding to the hydrophobicity are two palmitoyl chains on adjacent cysteine residues [7], a reported exception being canine SP-C, which has only one palmitoylated cysteine [9]. A lysine and an arginine in positions 11 and 12 of human SP-C (hSP-C) are the only two charged residues. These residues are thought to interact with the polar head groups of phospholipids [10] and neutralization of these charged residues leads to a decreased activity of the protein [11].

The function of the two palmitoyl chains on SP-C remains unclear. In general, palmitoylation of proteins can serve various purposes. It is particularly prevalent among proteins implicated in phenomena such as cellular adhesion, growth and signaling [12]. Palmitoylation of, for example, the α-subunits of G-proteins allow their attachment to the plasma membrane where they can exert their biological function [13,14]. However, palmitoylation of integral membrane proteins is also observed. One family of integral membrane proteins that can undergo palmitoylation is the G-protein coupled receptor family. Here, palmitoylation is a reversible process that is directly linked to receptor activation or translocation (for review, see [15]).

Given the structure and hydrophobicity of SP-C, it seems unlikely that the palmitoyl chains serve any of the described functions. The extreme hydrophobicity of the protein implies that it is membrane associated. Furthermore, SP-C is purified mainly in its dipalmitoylated form [16,17] and reversibility of palmitoylation has not been observed for SP-C.

In recent reports some effects have been found of depalmitoylation of SP-C. The orientation of depalmitoylated SP-C in a phospholipid monolayer is altered [18] and the surface activity in Wilhelmy plate experiments is decreased [18,19]. Furthermore, Vandenbussche et al. [20] showed that the secondary structure of depalmitoylated SP-C has a lower α-helical content, but an increase in α-helical content has also been reported [18].

The palmitoyl chains of SP-C could have a function in cross-linking two membranes. In this way lipids, which are squeezed out during compression, could stay associated with the monolayer. This would make them readily available for insertion upon the next expansion. Indications for the existence of these reservoirs have been observed in vivo by electron microscopy of the alveolar lining layer in rabbit lung [21]. Furthermore, scanning force microscopy revealed the formation of SP-C rich lipid reservoirs in vitro which were formed during compression of a phospholipid/SP-C monolayer [22].

In the last ten years the knowledge about the structure of SP-C and its function has increased dramatically. However, the extreme hydrophobicity still makes the functional examination of SP-C a difficult task. Recombinant SP-C and mutant forms have been expressed in *Escherichia coli* [18,23]. This could lead to a more pronounced insight into the structure-function relationship of this protein. Furthermore, synthetic peptides with SP-C sequence similarity have been made [24,25]. The disadvantage of these methods is that, in both cases, the proteins have to be chemically palmitoylated to be comparable to naturally occurring SP-C. In addition, especially with the synthetic peptides, the correct folding of the proteins remains a problem.

In this report we describe the production of hu-

man SP-C in the baculovirus expression system. This eukaryotic system has been used extensively to express a variety of proteins and has the advantage that it is capable of many post-translational modifications, including palmitoylation. The expressed protein was characterized both structurally and functionally and was found to have characteristics comparable to those of porcine SP-C.

2. Experimental procedures

2.1. Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol) (POPG) were obtained from Avanti Polar Lipids (Alabaster, AL, USA), HEPES from Life Technologies (Paisley, UK), EDTA, calcium chloride ($CaCl_2$), chloroform ($CHCl_3$), dichloromethane (DCM), *n*-butanol and methanol (MeOH) from Baker Chemicals (Deventer, The Netherlands). Organic solvents were distilled before use. Methanol, acetonitrile and propan-2-ol for HPLC experiments were purchased from LAB-scan (Dublin, Ireland) and were all HPLC grade. Porcine SP-C (pSP-C) was isolated from lung lavage as described previously [26].

2.2. Construction of recombinant transfer vector and recombinant baculovirus

Recombinant virus was produced as follows using a TA cloning kit and a MaxBac 2.0 kit from Invitrogen (Carlsbad, CA, USA). cDNA encoding the mature form of human SP-C was amplified by polymerase chain reaction (PCR), using a plasmid containing full length cDNA encoding human proSP-C [27] as the template and 5′-ACCATGTTTGGCATT-CCCTGCTGCCCA-3′ and 5′-TCAGAGACCCATGAGCAGGGCTCC as left and right-hand primers, respectively. The left-hand primer was designed in such a way that the codon for the N-terminal amino acid in mature SP-C was preceded by the sequence ACCATG, which contains the required start codon ATG and approximates the most favorable context for initiation of translation [28]. The right-hand primer was designed to create a stop codon (TGA) immediately behind the codon for the C-terminal amino acid of mature SP-C.

The PCR product was cloned directly into the linearized pCR-Bac vector according to the manufacturer's instructions. Recombinant virus was generated by homologous recombination between the transfer vector and linearized Bac-N-Blue AcMNPV DNA in *Spodoptera frugiperda* 9 (Sf9) cells. Insectin liposomes were used to optimize transfection. Recombinant viruses were plaque purified, amplified and tested for their ability to express hSP-C.

2.3. Insect cell culture, expression and purification of recombinant SP-C

The Sf9 insect cell line was propagated in Grace's insect media (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum and 10 μg ml^{-1} gentamicin. The cells were grown in monolayers and infected with recombinant virus at a multiplicity of infection (MOI) of 5. Cells were harvested after 72 h, resuspended in water, and sonicated 4×20 s on ice at 6 W using a Soniprep 150 ultrasonic disintegrator (MSE Scientific Instruments, Crawley, UK). The hydrophobic components of the suspension were extracted with *n*-butanol (H_2O:*n*-butanol, 1:40, v:v). The butanol was removed by rotary evaporation, and the residue was dissolved in DCM:MeOH:0.1 M HCl (70:25:5, v:v:v). Insoluble particles were removed by centrifugation. Recombinant SP-C (rSP-C) was separated from lipids and viral proteins by Sephadex LH-60 exclusion chromatography. The purified protein was stored in DCM:MeOH (1:1, v:v). Protein concentration was determined by quantitative amino acid analysis, which was performed by Eurosequence (Groningen, The Netherlands) on a HP 1090 Aminoquant, using a 2 step pre-column derivatization with *o*-phthalaldehyde-3-mercaptopropionic acid for primary and 9-fluorenylmethylchloroformate for secondary amino acids [29].

(N-terminal) sequence analysis was performed by using an Applied Biosystems-Perkin-Elmer sequencer Model 476A (using gas-phase cycles) by the Sequence Center, Institute of Biomembranes, Utrecht University, The Netherlands.

2.4. Protein electrophoresis

Protein electrophoresis was performed by one dimensional Tricine/SDS-PAGE [30]. After the electrophoresis, proteins were stained with silver stain (Bio-Rad Laboratories, Richmond, CA, USA).

2.5. Circular dichroism

CD spectra were obtained using a Jasco J600 spectropolarimeter in a 1 mm cell at 25°C. Pure protein was dissolved in *n*-butanol at a concentration of 150 μg ml^{-1}. Four consecutive scans from 240 to 195 nm were averaged and the protein-free butanol spectrum was subtracted to yield the protein spectrum. The CD spectra of porcine SP-C and recombinant SP-C were analyzed for contributions from the secondary structure by fitting the experimental data to poly-L-lysine reference spectra for α-helix, β-sheet and random coil [31] and to β-turn as described by Yang et al. [32]. This set of reference spectra gave the lowest root mean square values as defined by Brahms and Brahms [33] and were all lower than 10.

2.6. Attenuated total reflection fourier transform infrared spectroscopy

ATR-FTIR spectra were recorded at room temperature on a Bruker IFS-55 spectrometer, equipped with a liquid nitrogen cooled mercury cadmium telluride detector with a nominal resolution of 2 cm^{-1}. Pure protein or protein reconstituted in lipids was spread on a germanium ATR plate (50×20×2 mm with an aperture angle of 45°) by slowly evaporating the sample under nitrogen. A total of 1024 scans were averaged and corrected for the spectrum of a clean ATR plate. 10 μg pure protein was spread directly from $CHCl_3$:MeOH (1:1, v:v) onto the ATR plate. For the reconstituted samples, 40 μg of protein and 400 μg lipid (DPPC:PG, 8:2, mol:mol) were dried under nitrogen and traces of solvent were removed by overnight lyophilization. The protein/lipid film was hydrated in 2 mM HEPES, pH 7.4 at a lipid concentration of 1 mg ml^{-1}. The sample was incubated at 45°C for 15 min and vortexed in the presence of small glass beads for complete resuspension. Hydrogen to deuterium (H/D) exchange of readily accessible amide protons was obtained by flushing the sample on the ATR plate, sealed in a plate holder, with D_2O saturated nitrogen for 1 h. The amide I′ band (1700–1600 cm^{-1}) of the deuterated spectrum was used for quantification of the secondary structure of the protein, using a Fourier self-deconvolution procedure followed by a least-squares iterative curve fitting. This method has been described in great detail elsewhere [20,34].

For determination of the orientation of the protein in the lipid matrix, spectra were recorded with parallel (∥) and perpendicular (⊥) polarized light with respect to the incident plane. The mean angle between the helix axis and the normal of the plate was calculated from the dichroic ratio $R_{ATR} = A_{\parallel}/A_{\perp}$ The orientation of lipid acyl chains with respect to the normal of the ATR plate was calculated from the dichroic ratio of the phospholipid $\gamma_w(CH_2)$ band at 1200 cm^{-1} [35].

2.7. Mass spectrometry

Recombinant SP-C samples were dissolved in DCM:MeOH (50:50, v:v). Positive mode electrospray mass spectra were obtained on a VG Platform II single quadrupole mass spectrometer. Aliquots of 10 μl of the rSP-C samples were infused into a mobile phase of MeOH:H_2O (90:10, v:v) and introduced into the electrospray source at a flow rate of 5 μl min^{-1}. Spectra were scanned at a speed of 10 s^{-1} for *m/z* 600–2200 with a cone voltage of 60 V and recorded and processed using the MassLynx software, version 2.0. Mass calibration was performed by multiple-ion monitoring of horse-heart myoglobin signals.

2.8. Depalmitoylation of recombinant SP-C

40 μg of rSP-C in DCM:MeOH (50:50, v:v) was dried under nitrogen. The dried protein was resuspended in 45 μl $CHCl_3$:MeOH (1:1, v:v) containing 0.5 vol% 1 M HCl. After resuspension, 10 μl 5 M hydroxylamine, pH 7 containing 50 mM DTT (Boehringer Mannheim, Germany) was added and the sample was incubated overnight at 37°C.

2.9. Reverse phase high performance liquid chromatography

Reverse phase HPLC was performed as described by Gustafsson et al. [16] with a few modifications. A 250×4 mm LiChrospher RP-18 endcapped column (Merck, Germany) with a 5 μm particle size was used. Samples were dissolved in 50 μl DCM:MeOH (1:1, v:v) for HPLC analysis. Proteins were eluted at a solvent flow of 0.7 ml min^{-1}, using a propanol gradient of 0–80% in MeOH:H_2O (48:32, v:v), with a constant amount of acetonitrile (20%) (ACN) and trifluoroacetic acid (0.1%) (TFA) (Serva, Germany). The eluted proteins were detected by an Evaporative Light Scattering Detector (Alltech, Varex MKIII, Alltech, Deerfield, IL, USA) using the manufacturer's instructions. Relevant peaks were pooled for mass spectrometry and analysis on SDS-PAGE.

2.10. Captive bubble surfactometry

The activity of rSP-C to insert lipids into the air/liquid interface was determined using a pressure driven captive bubble surfactometer. A detailed description of this method is published [36]. Briefly, a bubble (0.5 cm^2) was formed in subphase buffer (140 mM NaCl, 10 mM HEPES, 0.5 mM EDTA, 2.5 mM $CaCl_2$, pH 6.9) by injecting air (32 μl) into the sample chamber at 1.0 ata and 37°C. A stock solution of rSP-C:DPPC:POPG with a molar composition of 3:77.6:19.4 was prepared in $CHCl_3$:MeOH (1:1, v:v). From this stock solution 0.05 μl (25 nmol lipids) was spread at the air/water interface using a glass syringe (7000.5, blunt tip, Hamilton, Switzerland). The subphase was stirred for 60 min to enhance desorption of solvent, after which the sample chamber was perfused 30 min with 7 ml buffer. Subsequently, 30 μl SUVs (DPPC:POPG, 80:20 mol:mol) were injected into the subphase (final concentration 1 mg DPPC ml^{-1}) and stirring was continued for another 15 min. The bubble area was increased by sudden lowering of the pressure to 0.5 ata for 10 s. Subsequently, the bubble was cycled 5 times between two preset pressure values of 0.5 and 2.8 ata in 1 min, resulting in a dynamic compression and expansion of the air bubble. A video camera continuously monitored the shape of the bubble, from which the surface tension values were calculated.

Statistical analysis was performed on the raw data using repeated measures analysis of variance (MANOVA). Differences were considered significant when P values were lower than 0.001.

3. Results

3.1. Expression and purification of recombinant SP-C

High titer baculovirus stocks containing the cDNA of mature hSP-C were generated and used to infect insect Sf9 cells. A butanol extract of the cells was applied to an LH-60 exclusion column as described in Section 2. The LH-60 chromatogram is depicted in Fig. 1. The small middle peak elutes at the same position (approximately 12 h) as SP-C in a butanol extract of porcine surfactant. Control experiments using insect cells infected with wild type virus resulted in a similar chromatogram with the exception of this peak, suggesting that this is the recombinant product. SDS gel electrophoresis of this recombinant protein peak yielded a single band at the same molecular weight as porcine SP-C (Fig. 2). The total yield of recombinant protein from sixteen 150 cm^2 cell culture flasks was 300 μg. Experiments were per-

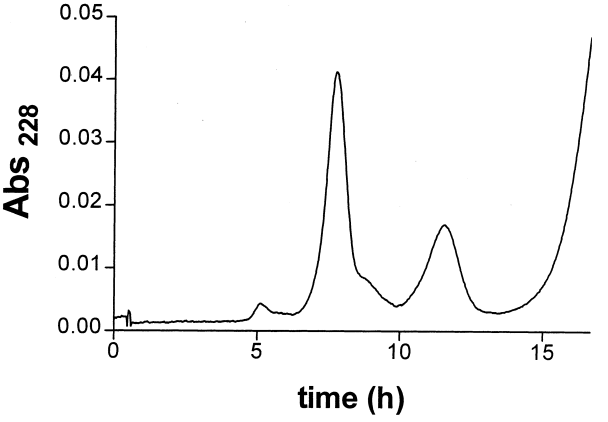

Fig. 1. Purification of recombinant SP-C by size exclusion chromatography. Sf9 cells infected with recombinant virus were collected, resuspended in water and sonicated. The butanol extract of this suspension was dried and applied to a Sephadex LH-60 column. Adsorption at 228 nm was monitored to detect the eluted components.

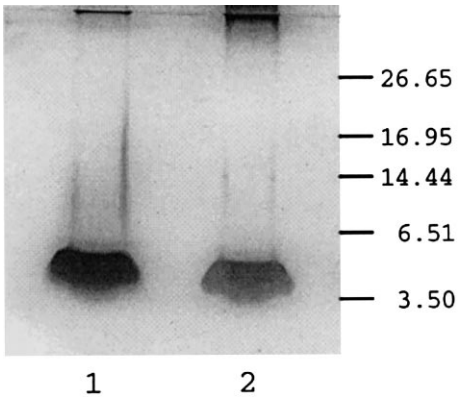

Fig. 2. SDS-Tricine gel of the recombinant SP-C. Lane 1: 10 μg porcine SP-C; lane 2: 3 μg recombinant SP-C.

formed to attempt to increase the expression. However, neither varying the multiplicity of infection between 1 and 10, nor harvesting cells after 48 or 96 h instead of 72 h, improved protein yield.

Next, we analyzed the recombinant protein by means of amino acid sequencing. The first 25 amino acids were analyzed and showed complete homology with hSP-C except for the starting methionine required to express the mature protein only. This recombinant protein will be referred to as rSP-C in the subsequent sections.

3.2. Circular dichroism

To obtain information on the secondary structure of rSP-C, CD was used. rSP-C and pSP-C were dissolved in butanol and a spectrum from 240–195 nm was recorded (Fig. 3). The spectrum shows that both proteins give rise to a typical α-helix shape with minima at 208 and 220 nm. Using the fitting program, an α-helical content of 73% was calculated for rSP-C.

3.3. ATR-FTIR

The regions between 1700 and 1600 cm^{-1} of the ATR-FTIR spectra of rSP-C and pSP-C are shown in Fig. 4. The observed peak represents the amide I′ band of the protein whose shape is closely related to the secondary structure of the protein. Both proteins clearly show a strong absorption in the α-helix region. The Fourier self-deconvolution curve-fitting procedure [20,34] was applied to the spectra which resulted in 73 and 72% α-helix for rSP-C and pSP-C, respectively. This is in good agreement with earlier presented values for SP-C using this method [20].

To determine the orientation of rSP-C in a lipid matrix, the recombinant protein was incorporated in multilamellar vesicles of DPPC:PG (8:2, mol:mol).

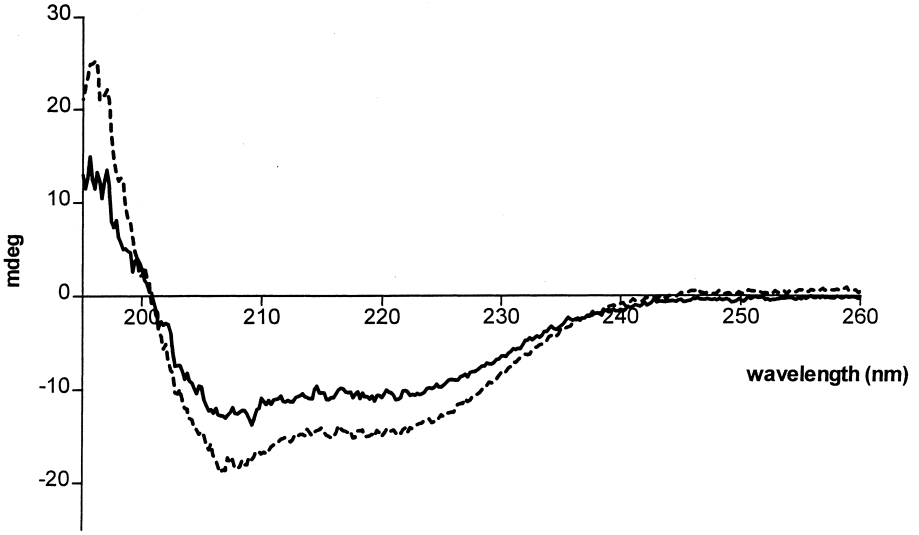

Fig. 3. CD spectra of recombinant- and porcine SP-C. rSP-C and pSP-C in DCM:MeOH (1:1) were dried under nitrogen and resuspended in *n*-butanol at a concentration of 150 μg ml^{-1}. Four consecutive scans from 240 to 195 nm were averaged and the protein-free butanol spectrum was subtracted to yield the protein spectrum. Dashed line: porcine SP-C, solid line: rSP-C.

Spectra were recorded with two orthogonal polarizations of the incident light, parallel (∥) and perpendicular (⊥) with respect to the incident plane (Fig. 5). The difference spectrum (∥−⊥) shows a clear positive deviation in the α-helix region, centered at 1657 cm^{-1}. A dichroic ratio $R_{ATR} = 2.4$ for the

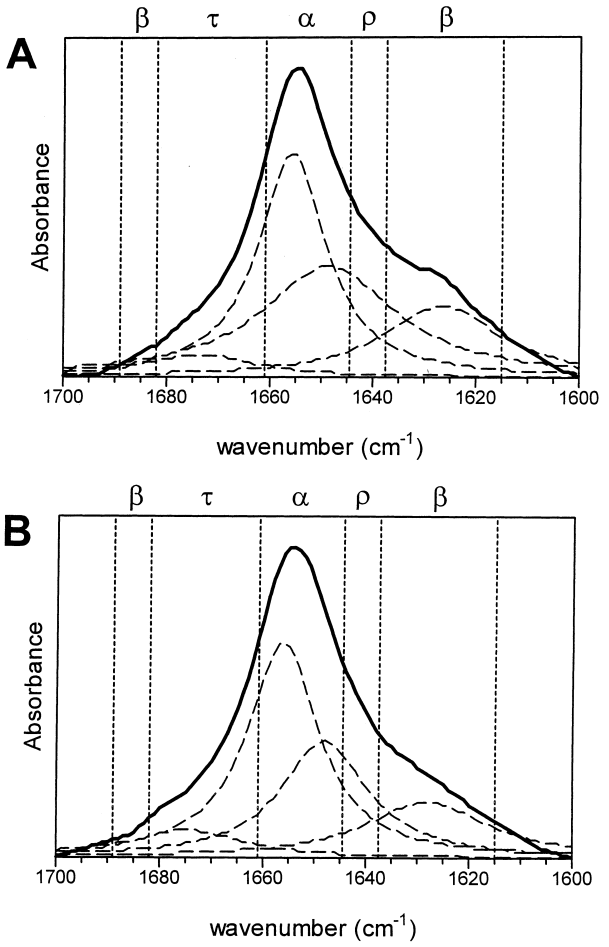

Fig. 4. Attenuated total reflection Fourier transform infrared spectrum of recombinant (A) and porcine (B) SP-C. 10 μg protein was spread on a germanium ATR plate by slowly evaporating the solvent under nitrogen. A total of 1024 scans were averaged and corrected for the spectrum of a clean ATR plate. The amide I′ absorption band, which contains information regarding the secondary structure of the protein, is shown. The secondary structure is quantified using a Fourier self-deconvolution procedure followed by a least-squares iterative curve fitting. The Lorentzian line shapes thus obtained are depicted under the original spectrum as dotted lines and represent a secondary structure contribution, depending on the position of their maximum. The vertical dotted lines limit the regions assigned to the different secondary structures. α: α-helix; β: β-sheet; τ: turns; ρ: random coil.

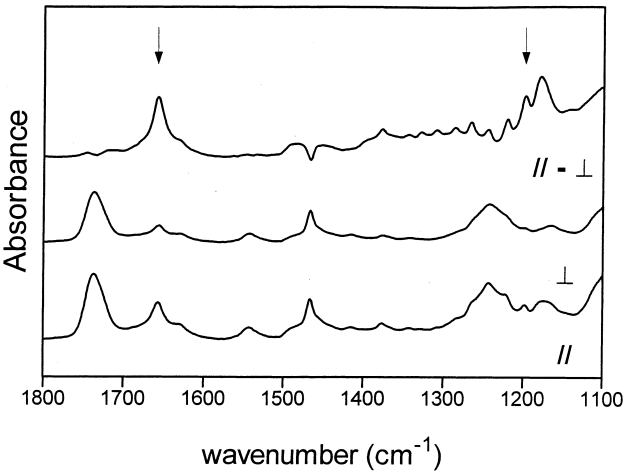

Fig. 5. ATR-FTIR spectrum of recombinant SP-C inserted in oriented lipid bilayers. 40 μg rSP-C and 400 μg lipid (DPPC:PG, 8:2, mol:mol) were dried under nitrogen and traces of solvent were removed by overnight lyophilization. The protein/lipid film was hydrated in 2 mM HEPES, pH 7.4 at a lipid concentration of 1 mg ml^{-1}. The dichroic spectrum (top tracing) represents the difference between the spectra recorded with parallel (∥) and perpendicular (⊥) polarized light with respect to the incident plane. The recorded spectra are drawn with identical ordinate scale, the dichroic spectrum is expanded threefold in the ordinate direction. The arrows indicate the protein amide I′ at 1657 cm^{-1} and the phospholipid $\gamma_w(CH_2)$ band at 1200 cm^{-1}.

α-helix component was calculated from the curve-fitting data. From this ratio a maximum tilt of 25° of the long α-helix axis with respect to the normal to the ATR plate was calculated. The average tilt of the lipid hydrocarbon chains with respect to the normal was found to be 22° ($R_{ATR} = 5.5$). This indicates a nearly parallel orientation of the SP-C helix to the lipid acyl chains, described before for pSP-C [20].

3.4. Mass spectrometry

The mass of the recombinant protein was determined with mass spectrometry, using electrospray ionization. The spectrum shows four peaks which can be divided into two groups of two (Fig. 6). The *m/z* peaks 965 and 1286 correspond to the 3^+ and 4^+ ions of a 3856 Da protein. This is close to the calculated mass of non-palmitoylated rSP-C, which is 3829 Da. A discrepancy between the calculated and measured *m/z* values (and other peaks with slightly higher masses in the spectrum) has been observed

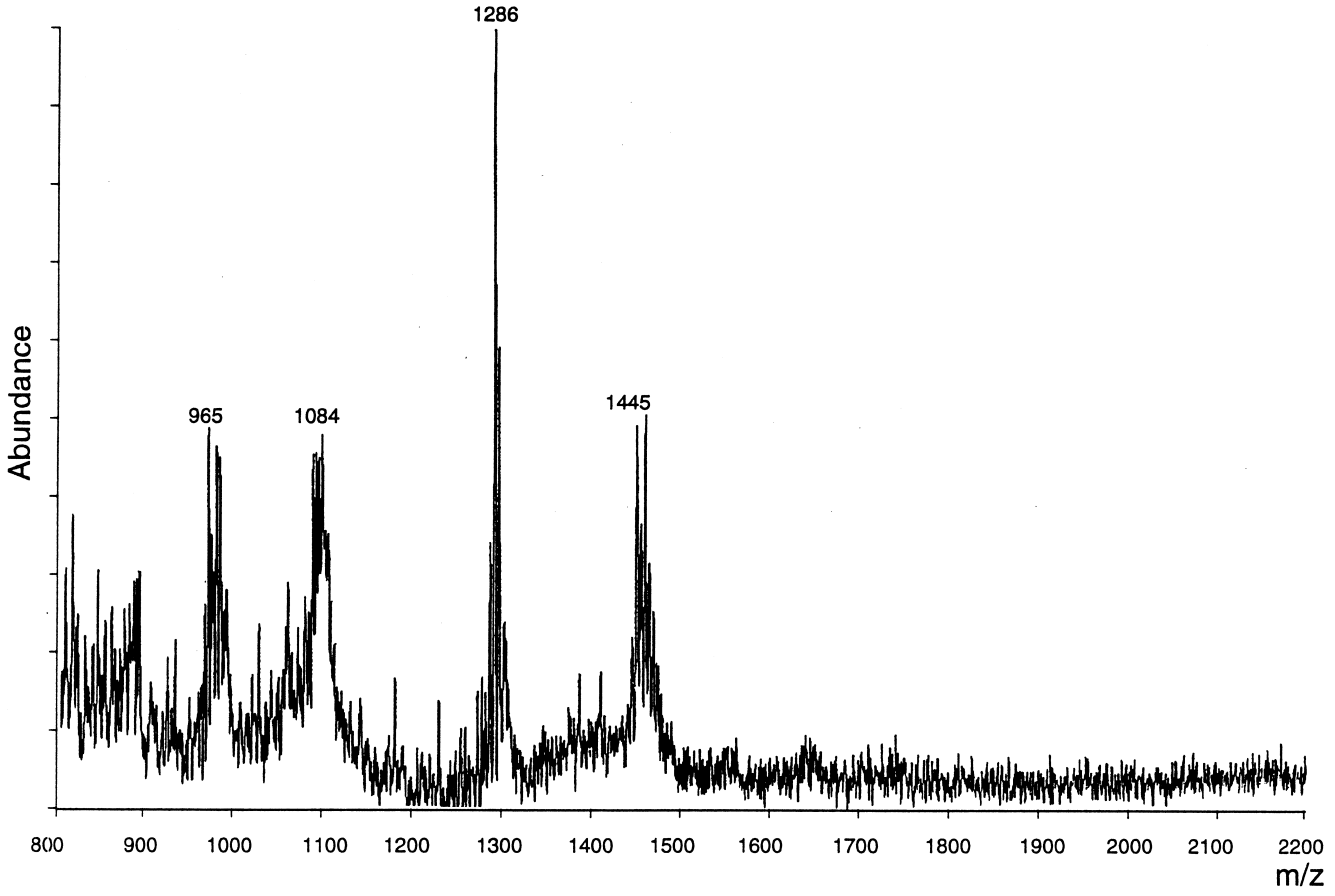

Fig. 6. Mass spectrum of recombinant SP-C. Recombinant SP-C was dissolved in DCM:MeOH (50:50, v:v) at a concentration of 320 μg ml^{-1}. An aliquot of 10 μl was infused into a mobile phase of MeOH:H_2O (90:10, v:v) and introduced into the electrospray source of the mass spectrometer at a flow rate of 5 μl min^{-1}. Spectra were scanned at a speed of 10 s^{-1} for *m/z* 600–2200.

before for SP-C in other mass spectrometry techniques [9] and is due to formation of sodium ion adducts (22 Da), C-terminal methyl ester (14 Da) and protonated forms. The *m/z* peaks at 1084 and 1445 correspond to the 3^+ and 4^+ ions of a 4332 Da protein. This is very close to the calculated mass of dipalmitoylated rSP-C (4305 Da) and exactly 2 times the mass of a palmitoyl chain (238 Da) higher than the other detected peaks. There are no clear peaks found that correspond to monoacylated rSP-C which would be expected around *m/z* values of 1024 and 1366. It should be noted that the spectrum cannot be interpreted quantitatively. The signal of a protein is strongly dependent on its ability to be ionized in the aerosolized sample and this behavior can be influenced by the hydrophobicity. One or two acyl chains difference can therefore lead to large differences in response signal.

3.5. Reverse phase high performance liquid chromatography

A C18 reverse phase column using a gradient of 2-propanol in methanol was used to further characterize rSP-C. Injection of rSP-C gave rise to three peaks with elution times of 16, 18 and 20 min (Fig. 7A) plus a non-symmetrical contribution at 14 min. All three peaks showed a clear SP-C band on SDS-PAGE, while the 14 min peak did not contain any detectable protein (result not shown). Part of this peak might arise from the detection method. An incomplete evaporation of the solvent mixture in the ELSD detector can cause such a signal. Protein-free samples did indeed give a signal at 14 min but subtraction of this baseline never completely removed the observed peak. A non-protein contamination which has not yet been characterized seems therefore

likely. Under the same conditions, native porcine SP-C gives rise to a minor peak at 19 min and a broad peak at 21 min, which are thought to be the mono- and dipalmitoylated forms of SP-C. De-acylation of native SP-C with neutral hydroxylamine causes a shift of both peaks to a retention time of 16 min (results not shown). Treatment of rSP-C with neutral hydroxylamine resulted in the disappearance of the last 2 peaks in the chromatogram (Fig. 7B), which suggested that these two peaks represented acylated forms of rSP-C, while the first peak represents an unacylated SP-C form. This was confirmed by mass spectrometry of the individual peaks which also revealed that both acylated peaks represent dipalmitoylated rSP-C (results not shown). Estimation of peak heights of the chromatogram (Fig. 7A) revealed that approximately 15% of the recombinant SP-C was in an acylated form.

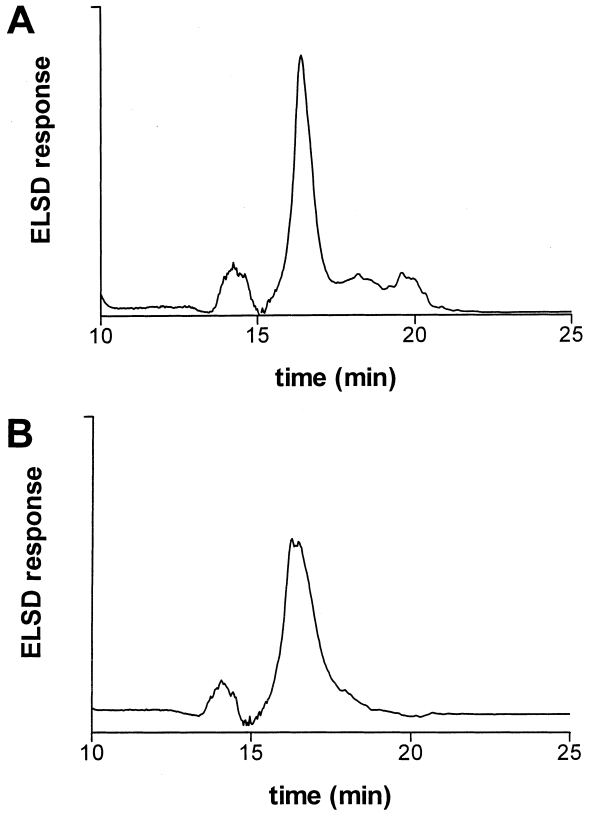

Fig. 7. Effect of neutral hydroxylamine treatment on the reverse phase high pressure liquid chromatography profile of recombinant SP-C. 30 μg rSP-C with and without neutral hydroxylamine treatment (as described in Section 2) was resuspended in 50 μl DCM:MeOH and applied to an RP18 column of 250× 4 mm. A propanol gradient from 0–80% was run in MeOH: H_2O (48:32, v:v) with a constant amount of acetonitril (20%) and trifluoroacetic acid (0.1%). Peaks were monitored by an evaporative light scattering detector. (A) HPLC chromatogram of 40 μg rSP-C. (B) HPLC chromatogram of 40 μg rSP-C treated with neutral hydroxylamine.

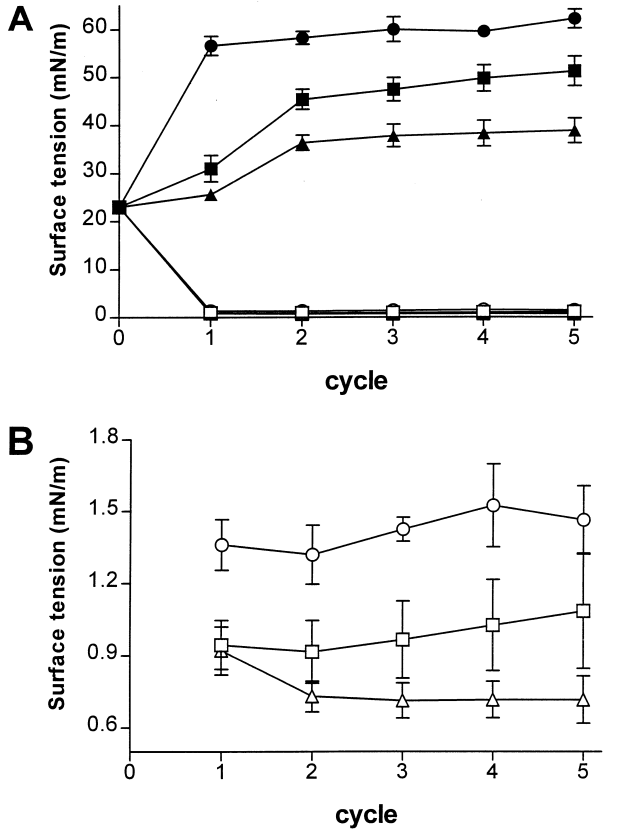

Fig. 8. Maximum and minimum surface tensions of SP-C in DPPC:POPG mixtures during dynamic cycling. DPPC:POPG (8:2, 25 nmol) containing 3 mol% porcine SP-C, 3 mol% rSP-C or no protein, was spread at the surface of a 32 μl air bubble at 37°C. SUVs of the same lipid composition were injected into the subphase (140 mM NaCl, 10 mM HEPES, 0.5 mM EDTA, 2.5 mM $CaCl_2$, pH 6.9) with a final concentration of 1 mg ml^{-1} DPPC. The bubble was expanded by reducing the system pressure to 0.5 ata. After 10 s adsorption, the bubble was cycled 5 times between 0.5 ata and 2.8 ata within 1 min. The bubble was monitored during the cycling procedure and from the shape of the air bubble the surface tension was calculated at minimum and maximum pressure. Data are mean ± S.E.M. ($n=3$, 7 and 3 for lipids only, rSP-C and pSP-C, respectively). Closed symbols represent γ_{max} values, open symbols represent γ_{min} values. Circles, lipids only; squares, 3% rSP-C, triangles, 3% porcine SP-C. (B) is an enlargement of the lower part of (A).

3.6. Captive bubble surfactometry

Monolayers of 3 mol% pSP-C or 3 mol% rSP-C in DPPC:POPG (8:2, mol:mol) and a protein-free DPPC:POPG (8:2, mol:mol) samples were spread at the bubble surface. This concentration of SP-C yields maximum activity, which is comparable to the activity reached for 0.2 mol% SP-B and 2 mol% SP-C mixtures in the same system [36]. The maximum and minimum surface tensions (γ_{max} and γ_{min}) of the samples during cycling are shown in Fig. 8A. There is a significant difference in the γ_{max} between the protein-free sample and the rSP-C sample, indicating that the recombinant protein has surface tension lowering activity. This was most noticeable in the first cycles. However, also a significantly lower activity for rSP-C compared to pSP-C was observed. In addition, pSP-C did not show the increase in γ_{max} upon cycling, which, although not statistically significant, was observed for the recombinant protein. In Fig. 8B, the γ_{min} values are shown in greater detail. Although all samples reach very low surface tensions (<2 mN m^{-1}), the same differences are seen as for the γ_{max} values. The protein-free sample never reaches a value lower than 1 mN m^{-1} while both pSP-C and rSP-C samples give values between 0.5 and 0.9 mN m^{-1}. Similar to γ_{max}, γ_{min} of rSP-C also gradually increases during cycling. However, only the difference between pSP-C and the protein-free sample reaches statistical significance.

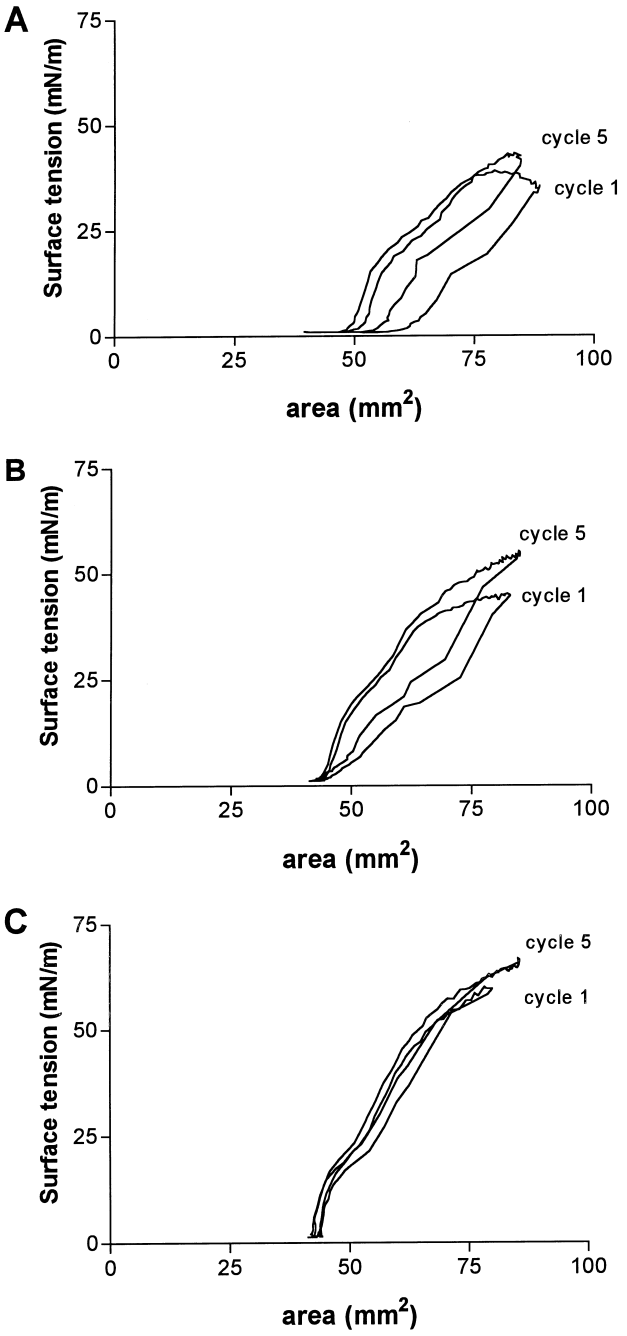

Fig. 9. Surface tension-area compression-expansion isotherms of SP-C in DPPC:POPG mixtures. A representative isotherm of each sample from the experiments described in Fig. 8 is shown. (A) 3% pSP-C; (B) 3% rSP-C; (C) lipids only. Only the first and last cycle are presented.

From the surface tension vs. area of the bubble isotherms depicted in Fig. 9, hysteresis is observed for the pSP-C and to a lesser extent for the rSP-C containing samples. Both samples also show overcompression when a pressure of 2.8 ata is applied to the system. The protein-free sample showed no hysteresis and was not overcompressed at these applied pressures.

4. Discussion

In this paper the expression of hSP-C in the baculovirus expression system and the characterization of this protein are reported. The recombinant protein was found to have similar structure and function as porcine SP-C and, in addition, was found to be partially palmitoylated. We utilized porcine SP-C as a positive control for the characterization of the recombinant SP-C, since all main characteristics of hSP-C are also present in pSP-C, including the palmitoylated cysteines at position 5 and 6. To our

knowledge, and despite a 5 amino acid difference, no structural or functional difference has been described between SP-C from these two species.

The secondary structure of the rSP-C consists of mainly α-helical structure, as determined by both CD and ATR-FTIR. An α-helical content of 73% was calculated from the ATR-FTIR spectra, which is in good agreement with the value for pSP-C (72%). It is also consistent with values described earlier for SP-C [8,20,37]. The low amount of palmitoylation of the recombinant protein did not seem to have an effect on the α-helical content, even though a lower helical content for non-palmitoylated SP-C has been described using FTIR as a detection method [20]. Why we do not see this phenomenon is unclear, but it has to be noted that another batch of rSP-C produced had a lower α-helix (51%) and a higher β-sheet (32%) content. Reconstitution in a lipid matrix of this particular batch led to a dramatic decrease of the β-sheet component, which is a known phenomenon for native SP-C. This indicates that, although the folding of this particular batch was not completely correct, the protein is not in an irreversibly denatured form.

The α-helix (amino acids 9–34) of SP-C is known to be a very stable and rigid structure [24]. This is somewhat amazing because it does not seem to contain typical α-helix amino acids. Valine and isoleucine (13 residues in the helix) in fact favor β-sheet conformation and the poly-valyl helix of SP-C is uncommon in membrane spanning proteins. Synthetic peptides with the mature SP-C sequence lack a high α-helical content and therefore a role for the precursor parts of SP-C in the folding process has been postulated [24]. However, the high α-helix content of the recombinant protein produced in the present study, indicates that in vivo a membrane environment and, possibly, additional factors are sufficient for correct folding. The uncommon rigid structure of the SP-C helix raises the possibility for a specific function of the poly-valyl helix. Its substitution by the membrane spanning α-helix of bacteriorhodopsin, or by a poly-leucine helix did not have an effect on the activity of the protein in spreading experiments [24,25]. However, its involvement in other possible functions of SP-C, like for example respreading during dynamic cycling, was not tested.

Mass spectrometry indicates that rSP-C is partially palmitoylated. Two populations of rSP-C were observed with masses of 3856 and 4332 Da. These are very close to the calculated masses of rSP-C without and with 2 palmitoyl chains (3829 and 4305 Da respectively). Judged from the HPLC chromatograms, 15% of the protein was susceptible to neutral hydroxylamine, causing a shift in the chromatogram. Neutral hydroxylamine disrupts thioester bonds, from which it can be concluded that the palmitoyl chains of rSP-C are thioester linked to the cysteine residues. The existence of two dipalmitoylated rSP-C subfractions in the HPLC chromatogram is most likely explained by a conformational difference between the two. At present, the nature of these different forms of rSP-C is unclear to us.

The expression of both the palmitoylated and non-palmitoylated form of proteins is not uncommon using the baculovirus expression system [38,39]. The fact that palmitoylation of rSP-C does occur, indicates that the N-terminal and C-terminal parts of proSP-C are not essential for this modification. No real palmitoylation motifs have been described yet, but evidently the mature protein contains information to induce palmitoylation of the protein. Nevertheless, the low palmitoylation degree of rSP-C might indicate that the precursor parts contain additional signals for palmitoylation. The precursor parts have also been proven to be necessary for post-translational targeting of SP-C in alveolar epithelial cells [6]. The produced recombinant product will probably not be exported to the secretory pathway of the insect cells and might therefore fail to come in contact with intracellular sites containing palmitoylthiotransferase activity. However, in a recent report by Grünewald et al., it was described that the palmitoylation of a dopamine receptor expressed in Sf9 cells was not influenced by the addition of brefeldin A [40]. This indicates that at least one of the possibly more palmitoylthiotransferase activity pools of these insect cells is within the ER or the early Golgi apparatus. There are more possible explanations for the low yield of palmitoylated rSP-C. It could, for instance, be due to the relatively late expression of the protein. The polyhedron promoter, which drives the expression, becomes activated in the very late phase of the baculovirus life cycle. Since the virus is lethal to the insect cell, many functions of the Sf9 cells might be shut down by the time SP-C is produced. For anoth-

er post-translational modification, glycosylation, it has been shown that using a promoter in the early phase of the virus life cycle could lead to a better glycosylated product [41]. The localization of rSP-C after expression could also interfere with palmitoylation. No palmitoylthiotransferase has been purified or cloned so far [42,43] and not much is known about the mechanism of palmitoylation. It is conceivable that the palmitoylthiotransferases cannot access the recombinant protein when it is embedded at a high concentration in membrane domains in the insect cells. Ways to increase the degree of palmitoylation are currently under investigation in our laboratory.

Besides being structurally similar to native SP-C, rSP-C was also found to be able to insert lipids into an air/liquid interface in captive bubble surfactometer experiments. Although it was less effective than pSP-C, rSP-C had the ability to lower the surface tension. During the first expansion and cycling, the surface tension of the bubble remained well below values reached for protein-free samples. The lower activity of rSP-C compared to pSP-C might be caused by several factors. The extra methionine added to the recombinant protein might affect the activity. In addition, there are indications for a non-protein contamination in the rSP-C samples, observed during the HPLC experiments. In what way the contamination could influence the captive bubble experiments is speculative. However, serum protein contamination has been shown to decrease the activity of surfactant in CBS experiments [21]. In our opinion the most likely cause of the lowered activity is the low amount (15%) of dipalmitoylated rSP-C. In comparable studies, contrasting results have been found concerning the role of the palmitoyl chains in surface tension lowering ability. Wang et al. [19] described a clearly decreased activity of depalmitoylated SP-C in Wilhelmy plate experiments. On the other hand, no, or very small differences have been observed in pulsating bubble experiments [44]. Interestingly, Qanbar et al. [45] described a clear effect of the palmitoyl chains in CBS studies. Based on studies with adsorbed films (instead of spread films used in our experiments), these authors postulated that non-palmitoylated SP-C is, besides being less active in adsorption, less capable of keeping a reservoir of lipids associated to the monolayer. Washing of the subphase followed by an overcompression of the bubble and a subsequent expansion led to significantly higher surface tension values for the samples containing the non-palmitoylated SP-C. Our results are in agreement with this suggested role of dipalmitoylated SP-C in lipid reservoir formation. Upon each cycle, the maximum surface tension of the samples containing rSP-C increases, which suggests that fewer lipids are directly available to be inserted into the lipid monolayer, despite the abundance of lipid vesicles in the subphase. This effect is not observed for pSP-C with more than 95% of its cysteines palmitoylated.

The view that SP-C contributes to the formation of a surface-associated lipid reservoir is consistent with earlier work from Oosterlaken-Dijksterhuis et al. [26], who reported that SP-B and SP-C promote insertion of lipids from vesicles associated to a preformed phospholipid monolayer. These associated vesicles were resistant to extensive flushing, even in the absence of calcium ions, showing that they were tightly bound to the monolayer.

Recently new reports describing the formation of reservoirs associated to the monolayer have been published [22]. In these experiments a monolayer of lipids and SP-C was spread in a Wilhelmy balance. Decreasing the surface area resulted in a collapse of the monolayer. The collapse occurred in domains rich in SP-C and the formation of bilayer lipid structures under these domains was visualized by fluorescence techniques and scanning force microscopy. The latter technique showed that phospholipid bilayers were stacked in these structures and piles of up to four bilayers were found. These stacks were not found in collapsed protein-free lipid monolayers. Upon expansion of the surface area these lipid stacks were inserted into the monolayer again. Indications for the occurrence of the described structures in vivo were recently reported [21]. Electron micrographs of the alveolar lining layer in rabbit lung showed sites with multilayers of lipids at the interface, as well as sites with amorphous material.

The captive bubble surfactometer has been proven to be a powerful tool in determining the activity of surfactant preparation [45–47]. All these experiments have been performed using absorbed films. The use of spread films instead of adsorbed films has very recently been described [36]. In these studies, a few

differences were observed between the two types of surface film preparation. Spread films (containing 2% SP-C and 0.2% SP-B on a weight basis) reached low surface tensions during the first dynamic cycle while adsorbed films (from lipid/SP-B/SP-C vesicles with the same composition) required 3 or more cycles. The adsorbed films on the other hand had lower γ_{max} values during dynamic cycling. It is therefore hard to compare the present experiments with spread films directly to experiments described with adsorbed films of SP-C/lipid mixtures [45], also because different concentrations of proteins (1% vs. 3% SP-C) and lipid composition were used. Comparison of the 3% pSP-C with the SP-B/SP-C (0.2/2%) mixtures in spread films [36] shows high similarities in several aspects such as area reduction needed to reach low surface tensions, hysteresis and overcompression-respreading kinetics. However, a more detailed study is required to assign the specific roles of SP-B and SP-C in several aspects of surface film dynamics.

The expression of a mixture of dipalmitoylated and non-palmitoylated SP-C offers an interesting opportunity to investigate the role of palmitoylation of SP-C even further. The baculovirus recombinant proteins offer the advantage that no chemical depalmitoylation (such as required when starting with SP-C isolated from animals) or chemical palmitoylation (rSP-C expressed in *E. coli*) has to be performed. In our system the two proteins are separated in the last step of purification, which means that they are treated exactly the same during the whole process and are, therefore, directly comparable. These studies, which are currently performed in our laboratory, will provide more insight into the role of the palmitoyl chains.

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