Biophysical activity of an artificial surfactant containing an analogue of surfactant protein (SP)-C and native SP-B

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Natural surfactant preparations containing phospholipids and the hydrophobic surfactant proteins B and C (SP-B and SP-C) are effective in the treatment of respiratory distress syndrome in premature infants. The limited supply, and the risk of infectious agents and immunological reactions have promoted the evaluation of synthetic peptides in surfactant preparations. However, the folding of synthetic SP-C into an α -helix is inefficient and α -helical SP-C analogues with Val $\rightarrow$ Leu substitutions form oligomers. In order to circumvent these problems we have synthesized an SP-C analogue, named SP-C(LKS), which differs from SP-C mainly by the exchange of most of the Val residues in positions 16–28 with Leu residues to promote an α -helical conformation, and by the introduction of Lys residues at positions 17, 22 and 27 in order to locate positive charges around the helical circumference and thereby avoid self polymerization. CD spectroscopy showed a spectrum typical for α -helical peptides and SDS/PAGE disclosed a single band. The biophysical activity of artificial surfactant preparations containing SP-C(LKS) and phospholipids, with and without native SP-B, was measured using a Wilhelmy balance and a pulsating bubble surfactometer.

SP-C(LKS) (3%, w/w) in a mixture of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)/phosphatidylglycerol/palmitic acid (68:22:9, by wt.) suspended in 150 mM NaCl, showed rapid spreading at the air-liquid interface and produced a surface tension of < 1 mN/m at minimum bubble size ($\gamma_{\min}$) and 42 mN/m at maximum bubble size ($\gamma_{\max}$) in the pulsating bubble surfactometer. The addition of 2% (w/w) SP-B to the preparation reduced the maximum surface tension to 33–35 mN/m, i.e. both $\gamma_{\min}$ and $\gamma_{\max}$ values were similar to those of natural surfactant preparations. Optimal *in vitro* characteristics were also obtained from a preparation containing SP-C(LKS), SP-B, DPPC and phosphatidylglycerol, i.e. when palmitic acid was omitted from the lipid mixture. SP-B containing surfactant preparations made up in Hepes buffer at pH 6.9, instead of in 150 mM NaCl, had similar biophysical activity provided that palmitic acid was omitted, but decreased activity in the presence of palmitic acid.

Key words: hydrophobic protein, mass spectrometry, protein separation, pulmonary surfactant, structure analysis.

INTRODUCTION

Pulmonary surfactant reduces surface tension at the air-liquid interface of the alveolar lining, preventing the lungs from collapsing at end expiration. Surfactant deficiency is a common disorder in premature infants and causes respiratory distress syndrome, which can be effectively treated with natural surfactants extracted from animal lungs [1]. The main constituents of these surfactant preparations are phospholipids such as 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), phosphatidylglycerol and the hydrophobic surfactant proteins B and C (SP-B and SP-C). The hydrophilic surfactant proteins SP-A and SP-D, which are C-type (Ca^{2+} -dependent) collagenous lectins and thought to act primarily in the host-defence system, are normally not included in the surfactant preparations due to the organic solvent extraction procedures employed.

SP-B and SP-C constitute only 1–2% of the surfactant mass, but are still able to exercise excessive improvements on surface activity, compared with pure lipid preparations [2,3]. The primary and secondary structures of SP-B and SP-C, and a tertiary structure of SP-C in solution, have been determined [4]. SP-B is composed of two identical polypeptide chains of 79 amino acids, connected with an interchain disulphide bridge [5,6]. Each

monomeric chain has three intrachain disulphide bridges and at least four amphipathic helices exhibiting one polar and one nonpolar face, through which SP-B may interact with two lipid bilayers and bring them into close proximity [7]. SP-C is a lipoprotein composed of 35 amino-acid residues with an α -helical domain between residues 9–34 [8]. The helix is composed mostly of valine residues and is embedded in a lipid bilayer and oriented in parallel with the lipid acyl chains [9]. Two palmitoyl groups are covalently linked to cysteine residues in positions 5 and 6 in the N-terminal part of the peptide [5]. The two conserved positively charged residues, arginine and lysine, at positions 11 and 12, possibly interact with the negatively-charged head groups of the lipid membrane, thus increasing its rigidity. The rigidity of the lipid-peptide interaction may be decreased towards the C-terminal end, since it contains small or nonpolar residues only, making this part potentially more mobile in a phospholipid bilayer. SP-C is thought to influence the thickness and fluidity of the surrounding lipids *via* the extremely stable polyvaline helix [4].

Surfactant preparations obtained from animal tissue are only available in limited amounts. They may furthermore contain infectious agents and induce immunological reactions. Therefore attempts have been made to create artificial surfactants [4,10],

Abbreviations used: DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; MALDI-TOF, matrix-assisted laser desorption ionization-time-of-flight; SP, surfactant protein; $\gamma_{\max}$, surface tension at maximum bubble size; $\gamma_{\min}$, surface tension at minimum bubble size.

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usually from synthetic lipids and hydrophobic proteins. Previous work has demonstrated difficulties in folding synthetic SP-C into the α -helical conformation necessary for optimal surface activity [11]. Substitution of the polyvaline segment for a polyleucine segment gave efficient α -helix formation but caused self polymerization [12]. We have now synthesized a polyleucine SP-C analogue, SP-C(LKS), where three leucines have been replaced with lysine residues in order to locate positive charges around the helical circumference, thus avoiding self polymerization. This polypeptide combined with lipids shows optimal surface activity *in vitro*, especially in combination with SP-B.

MATERIALS AND METHODS

Peptide synthesis and purification

An analogue of SP-C, SP-C(LKS) (Figure 1), was synthesized by use of stepwise solid-phase technology and *tert*-butoxycarbonyl chemistry [13] in an Applied Biosystems 430A instrument. Cleavage of the resin-peptide bond and deprotection of the side-chains were carried out in anhydrous hydrogen fluoride/methoxybenzene/dimethyl sulphide (10:1:1, by vol.) for 1.5 h at 0 °C. Protecting groups and scavengers were removed by repeated extractions with diethyl ether and the peptide was subsequently extracted from the resin by dichloromethane/trifluoroacetic acid (3:1, v/v) followed by rotary evaporation. The crude peptide extract was re-dissolved at a concentration of 100 mg/ml in chloroform/methanol (1:1, v/v) containing 5% H₂O. An aliquot of 10 mg was applied to a Sephadex LH-60 column (40 cm × 1 cm) in the same solvent [2]. Fractions of 2.5 ml were collected and absorbances at 214 and 280 nm were measured. Identification and quantification were performed by amino-acid analysis.

The 21-residue peptide KL₄ (KLLLLKLLLLKLLLLKLL-LLK) was synthesized and purified as described previously [14,15]. SP-B was purified from porcine lung tissue [2].

Human SP-C FGIPCCPVHLKRLLIIVVVVVLIVVVIVGALLMGL
 SP-C (Leu) -----SS--△-----LLLLLL--LLL-L-----
 SP-C (LKS) -----SS-----LKLLLLK--LLLKL-----

Figure 1 Amino-acid sequences and helical wheel presentations of SP-C and its analogues

The sequence of human SP-C is from [18] and that of SP-C(Leu) is from [12]. SP-C(LKS) is based on the primary structure of SP-C with the following changes: all Val residues at positions 16–28 (with the exception of position 17) have been replaced with Leu residues; Lys residues have been introduced at positions 17, 22 and 27; the palmitoylated Cys at positions 5 and 6 have been replaced with Ser. The helical wheel projections cover positions 8–35; the charged residues are circled and identified with their sequence locations.

Biochemical characterization

The purity of the peptide was checked by SDS/PAGE (Phast-system, Pharmacia, Uppsala, Sweden) and by reversed phase-HPLC, using a C₁₈ column and a linear gradient of 60% (v/v) aqueous methanol/0.1% trifluoroacetic acid and isopropanol/0.1% trifluoroacetic acid [16].

Molecular masses were determined by matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF)-MS (Laser-mat 2000, Finnigan MAT) calibrated with vasoactive intestinal peptide ($[M+H]^+$ 3326.8).

Peptide secondary structure was investigated using CD spectroscopy (JASCO-720 spectropolarimeter, JASCO, Tokyo, Japan). After solubilization with trifluoroethanol, spectra were recorded from 260 to 184 nm with a scan speed of 20 nm/min and a resolution of 2 data points/nm. The residual molar ellipticity was calculated and expressed in kdegrees · cm² · dmol⁻¹. Molar ellipticities at 208 and 222 nm were utilized for estimating the content of helical structure [17].

Preparation of peptide/lipid mixtures

DPPC, phosphatidylglycerol and palmitic acid were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The lipids, dissolved in chloroform/methanol (98:2, v/v), were mixed in the proportions DPPC/phosphatidylglycerol/palmitic acid (68:22:9, by wt.) or DPPC/phosphatidylglycerol (7:3, w/w). Surfactant preparations were prepared by adding SP-C(LKS) alone, SP-C(LKS) and SP-B, or SP-C(LKS) and KL₄, to each of the lipid mixtures, at a total polypeptide:lipid weight ratio of 0 to 0.05. The mixtures were evaporated under nitrogen and resuspended at room temperature in 150 mM NaCl or 10 mM Hepes buffer, pH 6.9, containing 140 mM NaCl, 2.0 mM CaCl₂, at lipid concentrations of 10–80 mg/ml. Repeated freezing and sonication (50 W, 48 kHz) were performed until homogeneous suspensions were achieved. In some cases the final suspensions were incubated at 45 °C for 1 h.

Biophysical characterization

Surface spreading kinetics were measured at 32–34 °C with a Wilhelmy surface balance (Biegler, Vienna, Austria). Surface tension was monitored during 10 min using a platinum plate connected to a strain gauge and inserted 1 mm into a hypophase consisting of 20 ml of 150 mM NaCl in a teflon trough. The suspensions were added as droplets (total 1 mg of lipids) on to the hypophase, 4 cm from the platinum plate.

Dynamic surface tension was recorded using a pulsating bubble surfactometer (Surfactometer International, Toronto, Canada) at 37 °C during 50% cyclic compression of the bubble surface and at a frequency of 40 cycles per min. All measurements were performed for 5 min and at a lipid concentration of 10 mg/ml. The pressures at specific time intervals were measured and used to calculate surface tensions at minimum (γ_{min}) and maximum (γ_{max}) bubble size.

RESULTS

Primary, secondary and quaternary structure of SP-C(LKS)

SP-C(LKS) is based on the primary structure of human SP-C (Figure 1). Val residues in the positions 16–28 with the exception of position 17 were replaced with Leu residues. At positions 17, 22 and 27 Lys (K) residues were introduced in order to locate positively charged residues separated by approximately 120° around the helical circumference. This was expected to prevent

Figure 2 Purification of SP-C(LKS) on Sephadex LH-60

Approx. 10 mg of crude peptide extract was applied to a Sephadex LH-60 column (40 cm $\times$ 1 cm) and eluted in chloroform/methanol (1:1, v/v) containing 5% (v/v) 0.1 M HCl (solid line) or chloroform/methanol (1:1, v/v) containing 5% H₂O (dotted line). The flow rate was kept at 6–8 ml/h and fractions of 2.5 ml were collected. The absorbance at 214 nm (A_{214}) was determined after evaporating the chromatography solvents and resolubilizing the samples in methanol.

peptide oligomerization by formation of repulsive intermolecular ion pairs (Figure 1). The two palmitoylcysteine residues at positions 5 and 6 were substituted with Ser residues.

For removal of synthesis by-products the peptide was initially purified by Sephadex LH-60 exclusion chromatography with the solvent system chloroform/methanol (1:1, v/v) containing 5% (v/v) 0.1 M HCl (Figure 2). The mass spectrum of the purified peptide, however, revealed an $[M+H]^+$ ion at m/z 3864, i.e. 14 mass units higher than expected (Figure 3A). This indicated that a methyl ester had been formed at the C-terminus [16]. Since esterification of carboxylic acids in alcohol is catalysed by acid, we replaced HCl with water in the Sephadex LH-60 solvent system. In this solvent system the peptide eluted somewhat later but still separated from low-molecular-mass by-products (Figure 2). The mass spectrum of SP-C(LKS), purified in the absence of HCl, revealed an $[M+H]^+$ ion at m/z 3850 (Figure 3B) that was in agreement with an expected molecular mass of 3849.

Analytical HPLC of SP-C(LKS) revealed a sharp peak eluting at approx. 36% (v/v) 2-propanol, indicating a single and folded conformation of SP-C(LKS) [16]. Secondary structure investigations of SP-C(LKS) using CD spectroscopy showed a spectrum typical for α -helical peptides and from the 208 and 222 nm minima an α -helical content of approx. 75% was estimated. The secondary structure remained stable following stepwise dilution with H₂O to 12% (v/v) trifluoroethanol, provided that the peptide was solubilized in 100% trifluoroethanol.

SDS/PAGE of SP-C(LKS) showed a single band with somewhat slower electrophoretic mobility than native SP-C (Figure 4). The slower mobility of SP-C(LKS) may be caused by aberrant binding of SDS although dimerization of SP-C(LKS) can not be ruled out. In contrast SP-C(Leu), which lacks Lys in the helical

Figure 3 Mass spectra of SP-C(LKS) purified on Sephadex LH-60

The contents of the main peaks in the chromatograms of Figure 2 were analysed by MALDI-TOF-MS. The peptide eluted with (A) chloroform/methanol (1:1, v/v) containing 5% (v/v) 0.1 M HCl had an $[M+H]^+$ ion with m/z 3864, while that eluted with (B) the same organic solvents containing 5% H₂O had an $[M+H]^+$ ion with m/z 3850. Calibration was made against vasoactive intestinal peptide, $[M+H]^+$ 3326.8.

part, forms oligomers (Figure 4). Furthermore, SP-C(Leu)/lipid mixtures were difficult to solubilize at concentrations higher than 20 mg/ml [2], while it was possible to make an SP-C(LKS)/lipid mixture with a lipid concentration of 80 mg/ml and a poly-peptide:lipid ratio of 0.03.

Figure 4 Electrophoretic analysis of SP-C(LKS)

The purified peptide SP-C(LKS) was analysed by SDS/PAGE (20% gel) under non-reducing conditions. Lane 1, native porcine SP-C (2.4 µg); lane 2, SP-C(LKS) (2.4 µg); lane 3, SP-C(Leu) (7 µg); size markers are shown on the left.

Figure 5 Surface spreading of synthetic surfactant preparations

Spreading kinetics of 3% (w/w) SP-C(LKS) (■), 3% (w/w) SP-C(LKS) with the addition of 2% (w/w) SP-B (△), and phospholipids alone (○). All preparations were examined at a concentration of 10 mg/ml DPPC/phosphatidylglycerol (7:3, w/w) suspended in 150 mM NaCl. The recordings were obtained with a Wilhelmy balance and each data point is the mean of three different recordings.

Biophysical activity

Kinetic measurements of 3% (w/w) SP-C(LKS) in DPPC/phosphatidylglycerol (7:3, w/w), using the Wilhelmy balance, showed a rapid spreading with a surface tension of 28 mN/m after 3 s (Figure 5). The spreading was somewhat slower using 1% (w/w) SP-C(LKS) in the same lipid mixture (results not shown). The addition of 2% (w/w) SP-B did not significantly change the spreading velocity or equilibrium surface tension (Figure 5). No improvements were observed after incubation of the mixture for 1 h at 45 °C (results not shown). Similar results were obtained using DPPC/phosphatidylglycerol/palmitic acid (68:22:9, by wt.) as the lipid mixture (results not shown).

In the pulsating bubble surfactometer 3% (w/w) SP-C(LKS) in DPPC/phosphatidylglycerol/palmitic acid (68:22:9, by wt.)

produced a $\gamma_{\min}$ of < 1 mN/m while a $\gamma_{\min}$ of 9–14 mN/m was observed with 3% (w/w) SP-C(LKS) in DPPC/phosphatidylglycerol (7:3, w/w; Table 1). The $\gamma_{\max}$ was 40 mN/m in both cases. The addition of 2% (w/w) SP-B gave $\gamma_{\max}$ values of 31–33 mN/m and $\gamma_{\min}$ values of 0–2 mN/m for both lipid preparations. These values are very similar to those obtained with surfactant preparations isolated from natural sources [19]. Incubation of the preparations at 45 °C for 1 h had no significant effect on surface activity (Table 1). Decreasing the amount of SP-B to 0.5% (w/w) in DPPC/phosphatidylglycerol (7:3, w/w) containing 3% (w/w) SP-C(LKS) tended to increase $\gamma_{\min}$, although the results did not reach statistical significance (Table 1). In contrast to SP-B, the addition of 2% (w/w) KL₄ [14] to 3% (w/w) SP-C(LKS) in DPPC/phosphatidylglycerol/palmitic acid (68:22:9, by wt.) did not reduce $\gamma_{\max}$, which remained relatively high at 41–42 mN/m.

Surfactant preparations suspended in 150 mM NaCl have a pH of 3.5–5.5. Lower pH values of 3.5–4.5 were observed in preparations containing SP-B. Since native SP-B is purified using acidified organic solvents [2] small amounts of acid may remain in the preparations. Near physiological pH was obtained by suspending the surfactant preparation in HEPES buffer, pH 6.9, containing 140 mM NaCl and 2 mM CaCl₂ (Table 2). Compared with the corresponding preparations in unbuffered saline there were no changes in $\gamma_{\max}$ or $\gamma_{\min}$ when DPPC/phosphatidylglycerol (7:3, w/w) was used as the lipid mixture. However when palmitic acid was included in the lipid mixture both $\gamma_{\max}$ and $\gamma_{\min}$ increased at the higher pH (Table 2).

DISCUSSION

The main component of pulmonary surfactant, DPPC, has a melting temperature of approximately 41 °C, and therefore does not spread properly on an aqueous subphase at normal body temperature. DPPC must be combined with other lipids and with specific surfactant proteins to decrease the melting temperature and to generate a stabilizing surface film in the terminal airspaces. The hydrophobic proteins SP-B and SP-C both enhance the biophysical activity of lipid mixtures mimicking the composition of natural surfactant. The principal role of SP-C in this context is probably to enhance spreading of the surfactant material, and this important biophysical property apparently depends on the α -helical conformation of the peptide, rather than on any specific amino-acid sequence [11].

Synthetic SP-C analogues containing the characteristic polyvaline sequence may not fold like the native peptide, and consequently do not interact properly with the surfactant lipids. To circumvent this problem, several attempts have been made to modify the sequence, e.g. by replacing all helical Val residues in native SP-C with Leu residues (Figure 1) which strongly favour α -helical conformation. This transmembranous analogue, SP-C(Leu), induced excellent spreading at an air-liquid interface when combined with DPPC/phosphatidylglycerol/palmitic acid (68:22:9, by wt.). However, the $\gamma_{\max}$ value was significantly higher than that of native surfactant. Furthermore, it was not possible to prepare lipid/peptide mixtures of higher concentrations than approx. 20 mg/ml, which was probably due to the formation of peptide oligomers [12]. Other workers have synthesized bioactive polyleucine SP-C analogues of different lengths, and neither self-oligomerization nor problems in producing samples of high lipid concentration were reported [20]. Difficulties in handling native SP-C and the self-polymerization phenomena urged us to introduce three Lys residues into the SP-C(Leu) sequence, thus spreading positive charges around the helical circumference (Figure 1).

Table 1 Surface properties of synthetic surfactant preparations in normal saline

The samples were measured directly after preparation or after a 1 h incubation at 45 °C at a concentration of 10 mg/ml phospholipids suspended in 150 mM NaCl. Preparations containing SP-C(LKS) and lipids were approx. pH 5.5 while preparations containing SP-B were pH 3–4. The recordings were obtained with a pulsating bubble surfactometer at 37 °C and 50% surface compression at a rate of 40 per min. Values are the mean (SD) of three to five measurements. PG, phosphatidylglycerol; PA, palmitic acid.

Surfactant preparation				Surface tension (mN/m)					
				7.5 s		1 min		5 min	
				$\gamma_{\min}$	$\gamma_{\max}$	$\gamma_{\min}$	$\gamma_{\max}$	$\gamma_{\min}$	$\gamma_{\max}$
SP-C(LKS) (%, w/w)	SP-B (%, w/w)	Phospholipids	Incubation						
3	—	DPPC/PG/PA	—	< 1	41(1)	< 1	41(1)	< 1	41(0)
3	—	DPPC/PG/PA	45 °C	< 1	41(1)	< 1	41(1)	< 1	41(1)
3	2	DPPC/PG/PA	—	< 1	33(2)	< 1	33(2)	< 1	33(2)
3	2	DPPC/PG/PA	45 °C	< 1	34(1)	< 1	34(2)	< 1	35(2)
3	—	DPPC/PG	—	12(5)	39(5)	14(4)	42(4)	9(5)	42(2)
3	—	DPPC/PG	45 °C	8(3)	35(5)	9(3)	39(5)	6(4)	42(3)
3	2	DPPC/PG	—	2(1)	31(1)	2(1)	31(3)	2(1)	33(1)
3	2	DPPC/PG	45 °C	3(3)	29(3)	1(1)	33(2)	1(0)	36(1)
3	1	DPPC/PG	—	< 1	24(4)	1(2)	26(4)	< 1	31(1)
3	0.5	DPPC/PG	—	4(2)	29(1)	4(3)	29(2)	3(1)	34(2)

Table 2 Surface properties of synthetic surfactant preparations in buffer

Preparations were examined at a concentration of 10 mg/ml phospholipids suspended in 10 mM Hepes buffer, pH 6.9, containing 140 mM NaCl and 2 mM CaCl_2 . The recordings were obtained with a pulsating bubble surfactometer at 37 °C, and 50% surface compression at a rate of 40 per min. Values are the mean (SD) of three to five measurements. PG, phosphatidylglycerol; PA, palmitic acid

Surfactant preparation				Surface tension (mN/m)					
				7.5 s		1 min		5 min	
				$\gamma_{\min}$	$\gamma_{\max}$	$\gamma_{\min}$	$\gamma_{\max}$	$\gamma_{\min}$	$\gamma_{\max}$
SP-C(LKS) (%, w/w)	SP-B (%, w/w)	Phospholipids							
3	—	DPPC/PG/PA		4(1)	44(2)	5(2)	47(2)	7(2)	50(1)
3	2	DPPC/PG/PA		3(3)	38(3)	4(4)	40(2)	3(3)	44(2)
3	—	DPPC/PG		15(3)	39(4)	16(2)	42(3)	13(3)	44(3)
3	2	DPPC/PG		2(2)	26(3)	1(1)	29(3)	< 1	35(1)

SP-C(LKS) acquires an α -helical conformation as shown by CD spectroscopy. The self-polymerization seen with SP-C(Leu) was not observed with SP-C(LKS) (Figure 4). Since both KL_4 [16], which like SP-C(LKS) contains Lys residues separated by approx. 120 °, and SP-C(Leu) [12] are transmembranous peptides, we assume that SP-C(LKS) also behaves as a transmembranous helical peptide in phospholipid bilayers. This assumption is indirectly supported by our observation that SP-C(LKS) induces very rapid spreading of surfactant lipids (Figure 5); a feature which is not observed with non-transmembranous polypeptides [12].

Purification of the SP-C(LKS) on Sephadex LH-60 in acidified chloroform/methanol favoured the formation of methyl esters, as detected by MS (Figure 3). Likewise, native SP-C purified in this way contains an esterified C-terminus [16]. By omitting acid in the purification procedure the formation of methyl esters could be avoided; the peptide eluted later during Sephadex LH-60 chromatography (Figure 2). If acid is omitted during the purification of endogenous SP-B and SP-C, using the Sephadex LH-60 system, then there is a loss in separation, especially between SP-C and the phospholipids (M. Palmblad, J. Johansson, B. Robertson and T. Curstedt, unpublished work). Therefore, SP-B used in our peptide-lipid mixtures was purified with acid included in the size-exclusion step, and hence methyl ester formation would be expected.

Measurements of surface activity, with the pulsating bubble,

gave $\gamma_{\min}$ values < 1 mN/m for SP-C(LKS), SP-C(Leu) and KL_4 in DPPC/phosphatidylglycerol/palmitic acid suspended in 150 mM NaCl, but all preparations had a $\gamma_{\max}$ > 40 mN/m. These results are in agreement with those reported previously for SP-C(Leu) [12] and KL_4 [21]. The addition of SP-B to SP-C(LKS) in DPPC/phosphatidylglycerol/palmitic acid significantly decreased $\gamma_{\max}$ to approx. 33 mN/m which is comparable to the $\gamma_{\max}$ of surfactant from natural sources. This result fits a model where SP-B keeps two lipid layers in close proximity [4]. SP-B may bind subphase lipid vesicles close to the interfacial monolayer and thereby facilitate rapid respreading of lipids at an air-liquid interface during surface expansion. This concept is further supported by electron microscopy, and by measurements obtained with the captive bubble surfactometer [22], which indicate that natural pulmonary surfactant may generate a film containing multilayers of lipids. It is possible that SP-B, or an analogue of this protein, needs to be included in a fully active artificial surfactant. The structure of Natural Killer (NK)-lysin, a basic polypeptide of 78 amino-acid residues which is homologous to SP-B [7], has recently been determined by NMR spectroscopy [23]. Since NK-lysin is a monomeric water-soluble molecule, features which are not applicable to SP-B, it is difficult to deduce structural features of membrane-bound SP-B from these data. Thus the rational design of SP-B analogues will have to wait for further data on the structure of SP-B in a lipid environment. It has been suggested that KL_4 is an SP-B analogue

[14]. When KL_4 was added to SP-C(LKS) in DPPC/phosphatidylglycerol/palmitic acid, $\gamma_{\max}$ remained at approx. 42 mN/m, indicating a difference in action between SP-B and KL_4 . This agrees with previous results showing that KL_4 is a transmembranous α -helix and thus structurally resembles SP-C rather than SP-B [15].

SP-C(LKS) in DPPC/phosphatidylglycerol had a $\gamma_{\min}$ of approx. 10 mN/m during cyclic compression, which is significantly higher than the corresponding measurements obtained with palmitic acid added to the mixture (Tables 1 and 2). However, when SP-B was added to SP-C(LKS) in DPPC/phosphatidylglycerol, $\gamma_{\min}$ was approx. 2 mN/m and $\gamma_{\max}$ approx. 31–33 mN/m, which is similar to the values found for natural surfactant. The surfactant preparations containing SP-B have a pH between 3–4 in 150 mM NaCl. By increasing the pH to 6.9, using a Hepes buffer, palmitic acid becomes negatively charged while the charges of the phospholipids and proteins are largely unchanged. The high $\gamma_{\max}$ in the preparation containing SP-B, SP-C(LKS) and the phospholipid mixture DPPC/phosphatidylglycerol/palmitic acid in Hepes buffer may be due to the interaction of the negatively charged palmitic acid with the positively charged residues on SP-B or SP-C(LKS), although SP-B appears to interact preferably with phospholipids rather than ionized fatty acids [25]. Palmitic acid may influence surface properties of surfactant films by introducing additional negative charges and/or by producing changes in membrane morphology. Palmitic acid has been widely used in surfactant preparations [24], but the natural abundance of palmitic acid in pulmonary surfactant is less than a few percent and our present data indicate that it is not essential for optimal *in vitro* activity.

In conclusion, we have shown that surfactant preparations with seemingly optimal *in vitro* properties can be obtained with SP-C(LKS), SP-B, DPPC and phosphatidylglycerol. SP-C(LKS) can be synthesized in large amounts and efficiently folds into an α -helix. Further studies will be focused on identifying SP-B analogues that can be obtained in large quantities, and on elucidating the roles of the SP-C palmitoyl groups for surfactant activity.

This work was supported by the Swedish Medical Research Council (Projects No 3351 and 10371), Konung Oscar II's Jubileumsfond and the Research Funds of the Karolinska Institute.

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