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PATENTE: PEPTIDOS SINTETICOS QUE TIENEN ACTIVIDAD SUPERFICIAL Y EL
USO DE LOS MISMOS EN LA PREPARACIÓN DE UN TENSIOACTIVO ARTIFICIAL

SOLICITANTE: CHIESI FARMACEUTICI S.p.A.

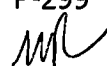
ASUNTO: CONTESTACION AUTO

DILIA RODRIGUEZ D' ALEMAN, en mi condición de apoderada de la sociedad solicitante, en esta oportunidad me permito dar respuesta a su auto notificado por estado de 28 de Octubre de 2004 y para ello anexo el siguiente documento:

- Copia de la publicación TAKEI TSUNETOMO ET AL. : " THE SURFACE PROPERTIES OF CHEMICALLY SYNTHETIZED PEPTIDES ANALOGOUS TO HUMAN PULMONARY SURFACTANT PROTEIN SP-C " BIO PHARM BULL (1996) 19 (10) 1247-1253, XP002139845, citada como anterioridad por la autoridad de búsqueda internacional y solicitada por su oficina para que pueda realizarse un análisis absoluto de la invención correspondiente a la presente solicitud.

Ruego a Ud. Continuar con el presente trámite.

Señor Jefe,


DILIA RODRIGUEZ D' ALEMAN
MR
P-299


The Surface Properties of Chemically Synthesized Peptides Analogous to Human Pulmonary Surfactant Protein SP-C

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Tsunetomo TAKEI^{*a,1)} Yohichi HASHIMOTO,^a Toshimitsu AIBA,^b Kaoru SAKAI,^b and Tetsuro FUJIWARA^c

Department of Biochemistry, Faculty of Science, Saitama University,^a 255 Shimo-okubo, Urawa 338, Japan. Research Laboratories, R&D Division, Tokyo Tanabe Co., Ltd.,^b 2-33-3 Akabanebiki, Kita-ku, Tokyo 115, Japan and Department of Pediatrics, Iwate Medical University,^c 19-1 Uchimaru, Morioka 020, Japan.

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A number of peptides with different lengths corresponding to various regions of human pulmonary surfactant protein SP-C were synthesized and their activity evaluated to improve *in vitro* surface activities and *in situ* lung pressure-volume characteristics of a ternary lipid mixture composed of dipalmitoylphosphatidylcholine, phosphatidylglycerol and palmitic acid (75:25:10, w/w). SP-C (1-35), a synthetic peptide with the entire length of human SP-C, and some other peptides with various lengths of its partial sequences were remarkably active. All of these peptides shared a common core sequence of (C)CPVHLKRLIVVVVVLIVVVIVGAL(L). Any deletion in this core sequence resulted in reduction of activity of the peptide. SP-C (5-31) and SP-C (6-32), the minimum peptides containing the core sequence, were combined with the ternary lipid mixture at the final peptide concentration of 2% (w/w) into synthetic surfactants which showed excellent properties comparable with those of Surfacten[®], a commercially available modified bovine lung surfactant. In a Langmuir-Wilhelmy surface balance, the synthetic surfactant containing SP-C (6-32) spread and adsorbed quickly to reach a surface tension of 30.8 mN/m at 30-s spreading time and 41.2 mN/m at 1-min adsorption time, respectively; the presence of SP-C (6-32) significantly prevented the decrease of surface activity of the ternary lipid mixture during dynamic compression-expansion cycles. Furthermore, tracheal instillation of the synthetic surfactant containing SP-C (6-32) at the dose of 50 mg of phospholipids/kg improved lung pressure-volume characteristics of immature rabbit neonates to a level similar to that of mature neonates at term.

Key words pulmonary surfactant; surfactant protein-C (SP-C); synthetic peptide; surface activity

Avery and Mead clearly demonstrated that infants dying of respiratory distress syndrome had a deficiency of pulmonary surfactant.²⁾ In 1980 Fujiwara and co-workers reported the first successful administration of modified bovine lung surfactant given as a liquid and instilled into the trachea of preterm infants with respiratory distress.³⁾ Since then surfactant replacement therapy has become standard care for neonates with infant respiratory distress syndrome (IRDS),⁴⁾ and has triggered interest in its clinical potential for other forms of lung diseases including acute lung injury in adult patients.⁵⁾

Surfactant preparations currently used in clinical practice are divided into two types: those that are modified natural surfactants containing specific surfactant proteins and isolated from mammalian lungs, and those made from synthetic lipid mixtures. *In vivo* and *in vitro* studies indicated that the presence of surfactant proteins is critical for surfactant function.^{6,7)} Clinical trials with modified natural surfactants compared with the synthetic lipid surfactant give some indication that surfactant proteins may be important constituents of therapeutic surfactants in some infants.⁸⁻¹⁰⁾ It has been indicated that the effects of surfactant proteins on the degree of surfactant inactivation may be clinically relevant.⁸⁻¹⁰⁾

Pulmonary surfactant contains two hydrophilic, collagen-like proteins, SP-A and SP-D, and two extremely hydrophobic, amphipathic proteins, SP-B and SP-C.¹¹⁻¹³⁾ These latter two specific peptide components observed in modified natural surfactants for clinical use are important in surfactant function.¹⁴⁾ *In vivo* studies indicated that they remarkably accelerate the spreading and absorption

of phospholipids to the air-water interface. In our early work^{15,16)} it was demonstrated that the biophysical and physiological properties of surfactant lipids were improved by adding a hydrophobic protein mixture of SP-B and SP-C isolated from bovine lungs.

SP-C of human is a 35-amino acid polypeptide that can be divided into two specific parts: a 23-residue hydrophobic C-terminal region rich in valine and leucine, and a hydrophilic N-terminal moiety bearing two conserved positive charges, lysine and arginine, and two adjacent cysteine residues that are stoichiometrically palmitoylated.¹⁷⁻²⁰⁾ As observed in SP-B, addition of SP-C alone to surfactant lipids enhances their surface activity *in vitro* and *in vivo*.^{21,22)} Synthetic analogues of human depalmitoyl SP-C mimic surface chemical and physiological properties of native surfactant peptide.^{21,23,24)} Data from these analog peptides indicated that surface activity of SP-C might depend on its α -helical configuration of hydrophobic C-terminal region and conserved hydrophilic amino acid residues at the N-terminal moiety.

In this study, we used a series of synthetic peptides with an entire sequence and a variety of truncated sequences of depalmitoyl human SP-C. These peptides were added to the ternary lipid mixture and their surface activity was evaluated with a Langmuir-Wilhelmy surface balance. We were interested in the structure-function relationships of these peptides and *in vitro* surface properties, e.g., whether the total amino acid sequence was necessary, or whether some regions or a only fragment was enough for the activity of SP-C. Peptide effects on respreading kinetics during dynamic compression-expansion cycles

* To whom correspondence should be addressed.

and on pressure-volume properties of surfactant-deficient preterm rabbit fetus were also investigated.

MATERIALS AND METHODS

Materials 9-Fluorenylmethyloxycarbonyl (Fmoc) ester of amino acids and other reagents using peptide synthesis were purchased from Kokusan Chemical Works, Ltd. (Tokyo, Japan). DL- α -Dipalmitoylphosphatidylcholine (DPPC) and phosphatidylglycerol (PG; sodium salt, prepared from egg yolk phosphatidylcholine) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and palmitic acid (PA) was from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Surfacten® (Surfactant TA) was a modified bovine lung surfactant containing a few percent proteins and about 95% lipids, and was supplied by Tokyo Tanabe Co., Ltd. (Tokyo, Japan). Exosurf® was a protein-free lipid surfactant composed of DPPC hexadecanol-tyloxapol (13.5:1.5:1, w/w), and was obtained from Burroughs-Wellcome Co. (Research Triangle Park, NC, U.S.A.). All other chemicals were of reagent grade.

Peptide Synthesis and Purification Peptides were synthesized by stepwise solid phase synthesis employing the Fmoc strategy in a solid phase multi-peptide synthesis system, KOKKU-San® (Kokusan Chemical Works, Ltd.), according to the method of Akagi *et al.*²⁵⁾ After cleavage of Fmoc protective group of the N-terminal amino acid residue by treatment with 20% piperidine in dimethylformamide, liberation of the peptide and removal of other protecting groups were carried out in a mixture of *m*-cresol-1,2-ethanedithiol-thioanisole-trifluoroacetic acid-trimethylsilylbromide (2:5:12:75:14, v/v). Resin was removed by filtration and diethylether was then added into the concentrated filtrate to precipitate the peptide. Peptide after cleavage was dissolved in trifluoroacetic acid-dichloromethane (5:95, v/v) containing 10 mM mercaptoethanol. From this solution, peptide was purified by HPLC on a 7.5 × 500-mm column of Asahipak GS-510 (Asahi Chemical Industry Co., Ltd., Kawasaki, Japan) which was eluted with the same solvent at a flow rate of 0.8 ml/min. Four-ml portions of eluate were collected and fractions containing purified peptide were pooled. The peptides were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fast atom bombardment mass spectrometry method using the mass analyzer model JMS-S102A (JEOL, Ltd., Tokyo, Japan). The composition of the purified peptide was determined by acid hydrolysis using 12 N HCl/trifluoroacetic acid (2:1, v/v) containing 5% (w/v) phenol at 150 °C for 1–72 h *in vacuo*. The hydrolyzate was analyzed on a LC-9A automatic amino acid analysis system (Shimadzu Co., Ltd., Kyoto, Japan). The amino acid compositions were calculated from the peak values of each amino acid obtained from the time course of their analysis.

The number of amino acid residues and fragment length of synthetic peptides correspond to the number in the published human SP-C, e.g., SP-C (1–35) corresponds to the full-length sequence of the depalmytoyl human SP-C.²⁶⁾

Native SP-C was isolated from an extract of bovine lung mince. A mixture of native SP-B and SP-C was isolated from a bovine lung extract containing phospholipids, neutral lipids, fatty acid and surfactant proteins as described previously.^{15,16)} The lung extract containing 1.4 g of phospholipids was dissolved in 4 ml of chloroform-methanol (2:1, v/v) and loaded on a column of Sephadex LH-20 (2.8 × 150 cm). The column was eluted with the same solvent system at a flow rate of 0.3 ml/min. Fractions of 3.6 ml were collected and were analyzed by SDS-PAGE. Proteins and phospholipids in the fractions were measured by Duley's method²⁷⁾ and King's method,²⁸⁾ respectively. Fractions containing SP-B and SP-C were dried and dissolved in chloroform-methanol-0.1 N HCl (20:10:1, v/v). Then, SP-C was separated from the mixture containing 19.9 mg of proteins and was purified to homogeneity by Sephadex LH-60 chromatography (2.48 × 90 cm) using the same solvent system at a flow rate of 0.3 ml/min in the manner described by Curstedt *et al.*¹⁴⁾ Fractions of 3.3 ml were collected and analyzed by SDS-PAGE. Purified SP-C was evaporated, dissolved in chloroform-methanol (2:1, v/v) and stored at –20 °C.

Preparation of Surfactant Peptide-Lipid Mixture Synthetic surfactant containing peptide was prepared in the following manner. Synthetic peptide, dissolved in a small volume of trifluoroacetic acid, was mixed with the ternary lipid mixture of DPPC-PG-PA (75:25:10, w/w) in a chloroform/methanol (2:1, v/v) solution so as to produce a peptide-lipid mixture containing 2% of peptide on a weight/weight basis relative to the phospholipids. The organic solvents were removed by evaporation. The peptide-lipid mixture was suspended in 10% ethanol solution, incubated at 40–45 °C for 15 min and then lyophilized to obtain a white powder. This admixture was dispersed in saline by sonication for 1 min in ice-cold water and was stored under nitrogen at –20 °C. The final concentration of phospholipids was 10 mg/ml.

Surfacten® and Exosurf® were also dispersed in saline at a phospholipid concentration of 25 and 13.5 mg/ml, respectively.

Langmuir-Wilhelmy Surface Balance Surface tension was measured with a modified Wilhelmy surface balance (Kyowa Co., Ltd., Tokyo, Japan) as described previously.¹⁶⁾ A sample of surfactant preparation containing 81 µg of phospholipids was spread on an air-water interface of saline (50 ml) in a Teflon trough (15 × 6 × 1.5 cm) and allowed to spread spontaneously for 3 min. Surface tensions at 30 and 120 s after spreading were monitored directly with a roughened platinum plate supported by the strain gauge. After 3 min, the resultant surface film was compressed and expanded from a maximum (54.0 cm²) to a minimum (21.6 cm²) surface area at 2.4 min/cycle. Cycling was continued until no further change was observed. Surface area at 10 mN/m obtained from the fourth cycle of a dynamic surface-tension diagram was expressed as percent of the area. Subphase temperature was controlled during measurement at 37 ± 1 °C.

Dynamic respreading after compression past collapse was measured by the method of Turcotte *et al.*²⁹⁾ The sample was spread from hexane-ethanol (9:1, v/v) on a

122 3
170

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50-ml saline subphase. The surface concentration used was $13 \text{ \AA}^2/\text{molecule}$. After 30 min, dynamic surface tension-area diagrams were recorded as above.

Surface absorption was measured in a round Teflon trough (5 cm in a diameter and 2 cm deep). While stirring at 60 rpm, a surfactant suspension containing 1.5 mg phospholipids was injected deep into 30-ml saline subphase. The change in surface tension was measured at 1, 3 and 5 min at $37 \pm 1^\circ \text{C}$.

Pressure-Volume Characteristics of Neonatal Lungs

Lung pressure-volume characteristics in premature rabbit fetus were measured by the method of Enhorning and Robertson³⁰ with several modifications. At 27 and 30 d gestation, a rabbit was anesthetized with sodium pentobarbital and fetuses were delivered by caesarian section. Without allowing to take a breath, the animal was weighed and the trachea was cannulated. A surfactant suspension containing 50 mg of phospholipids per kg body weight was instilled into the cannula which was then connected to a syringe and a water manometer. Air was put into a trachea at the flow rate of 0.8 ml/min using a syringe pump to increase pressure to 30 cm H₂O and expand the lungs. After 2 min, pressure was reduced to 0 cm H₂O at the same flow rate and the lungs were contracted. The fetuses were kept at $37 \pm 1^\circ \text{C}$. For each neonate studied with surfactant, two littermates were matched as control.

Data Analyses Statistical analyses were done using Student's unpaired *t*-test. All data are depicted as mean $\pm$ S.D. *p* values of less than 0.01 were considered to indicate a statistically significant difference.

RESULTS

Biophysical Activity of the Synthetic Peptides Analogous to Human SP-C A synthetic human SP-C, SP-C (1-35), and its truncated analogues are shown in Fig. 1. The spreading kinetics of each lipid-peptide mixture was evaluated by measurement of the surface tension after injection of the sample on an air-water interface, and then dynamic surface tension-area diagrams were recorded. The results of the surface activity (*i.e.*, the ability of the samples to reduce surface tension of film) of the lipid-peptide admixtures are summarized in Table 1. For comparison, the results of both clinically used surfactant preparations, Surfacten[®] containing SP-B and SP-C and protein-free Exosurf[®], and an admixture prepared with bovine native SP-C and lipids are also shown in Fig. 2.

The ternary lipid mixture consisting of DPPC/PG/PA (75:25:10, w/w) was utilized in the current investigation. This mixture was well characterized by Tanaka *et al.*¹⁶ with regard to surface properties and was used to investigate the influence of a lipid-associated polypeptide on dynamic surface activity of a lipid mixture. Spreading of this lipid mixture of DPPC/PG/PA was much slower. In a Langmuir-Wilhelmy surface balance, DPPC/PG/PA reached a surface tension of approximately 46 mN/m at 2-min spreading time (Table 1, Fig. 2). After spreading, films were compressed by 56.1% to reach a surface tension of less than 10 mN/m and had a minimum tension of 5.3 mN/m (Table 1, Fig. 2). Exosurf[®] also showed a very slow spreading (data not shown) and did not yield a

	1	10	20	30	35
SP-C(1-35)	FGIPCCPVHLKRL	IVVVVV	VLIVVV	VIVGALL	MGL
SP-C(5-35)	CCPVHLKRL	IVVVVV	VLIVVV	VIVGALL	MGL
SP-C(6-35)	CPVHLKRL	IVVVVV	VLIVVV	VIVGALL	MGL
SP-C(7-35)	PVHLKRL	IVVVVV	VLIVVV	VIVGALL	MGL
SP-C(1-32)	FGIPCCPVHLKRL	IVVVVV	VLIVVV	VIVGALL	
SP-C(1-31)	FGIPCCPVHLKRL	IVVVVV	VLIVVV	VIVGALL	
SP-C(1-30)	FGIPCCPVHLKRL	IVVVVV	VLIVVV	VIVGA	
SP-C(1-29)	FGIPCCPVHLKRL	IVVVVV	VLIVVV	VIVG	
SP-C(1-27)	FGIPCCPVHLKRL	IVVVVV	VLIVVV		
SP-C(1-16)	FGIPCCPVHLKRL	IV			
SP-C(4-30)	PCCPVHLKRL	IVVVVV	VLIVVV	VIVGA	
SP-C(5-31)	CCPVHLKRL	IVVVVV	VLIVVV	VIVGALL	
SP-C(6-32)	CPVHLKRL	IVVVVV	VLIVVV	VIVGALL	
SP-C(7-33)	PVHLKRL	IVVVVV	VLIVVV	VIVGALL	
SP-C(6-12)	CPVHLKR				
SP-C(13-32)		LLIVVVVV	VLIVVV	VIVGALL	
SP-C(6-20)	CPVHLKRL	IVVVVV			
SP-C(21-32)			VLIVVV	VIVGALL	

Fig. 1. Amino Acid Sequences of Synthetic Peptides Analogous to Human SP-C

minimum tension of less than 10 mN/m even with full compression (Fig. 3). In contrast with the ternary lipid mixture or Exosurf[®], reconstituted surfactant with bovine native SP-C and Surfacten[®] had excellent surface properties (Fig. 2).

It is clear that the bovine native SP-C and each of the synthetic peptides of an entire length and various regions of human SP-C molecule enhance the biophysical activity of surfactant lipids in varying and different degrees (Table 1). Deletions of N-terminal residues between 1-5 or C-terminal residues between 31-35 did not significantly affect biophysical activity of these peptides. Native SP-C, SP-C (1-35) and some other peptides, all of which contain a region of the central portion of the SP-C molecule, *i.e.*, residues 5 through 32 with a sequence of (C)CPVHLKRLIVVVVVVLIVVVIVGALL(L), significantly increased the spreading rates compared with the ternary lipid mixture (Table 1, $p < 0.01$); the surface tensions achieved after 30 s (about 30 mN/m) are comparable with those obtained under the same conditions with reconstituted surfactant with bovine native SP-C and Surfacten[®] (Fig. 2). After spreading, these lipid-peptide admixtures were compressed by only 30% to reach surface tensions below 10 mN/m. The surface activity of dynamic cycled films and their surface tension-area diagrams are equivalent to those of Surfacten[®] in which large collapse plateaus at the minimum tensions (1.2-5.4 mN/m) and rapid respreading with the low maximum tensions (27.2-36.2 mN/m) were observed (Fig. 3). Other peptides, all of which had some deletion(s) in this core sequence mixed with DPPC/PG/PA, showed rapid spreading, but surface tensions were significantly higher, 40-47 mN/m ($p < 0.01$ vs. native SP-C at 30-120 s after spreading). During dynamic cycles, the minimum tensions below 10 mN/m were still observed, whereas the maximum tensions tended to approach 40 mN/m. The area changes required to reach tensions below 10 mN/m were significantly increased to

Table 1 Effect of Synthetic Peptides Related to Human SP-C on Surface Activity of a Ternary Lipid Mixture of DPPC/PG/PA

Sample	n	Surface tension		Surface area at 10 mN/m (%)	Surface spreading	
		$\gamma_{\min}^{a)}$ (mN/m)	$\gamma_{\max}^{b)}$ (mN/m)		at 30 s (mN/m)	at 120 s (mN/m)
DPPC/PG/PA	5	5.3 ± 1.5	51.3 ± 7.1	43.9 ± 2.0	47.7 ± 2.8	46.3 ± 1.8
+SP-C(1—35)	5	1.2 ± 0.3 ^{c)}	33.3 ± 1.2 ^{d)}	75.3 ± 2.0 ^{d)}	28.3 ± 1.0 ^{d)}	27.2 ± 0.8 ^{d)}
+SP-C(5—35)	3	1.9 ± 0.4 ^{c)}	31.5 ± 2.1 ^{d)}	73.5 ± 1.9 ^{d)}	29.8 ± 1.3 ^{d)}	28.6 ± 1.1 ^{d)}
+SP-C(6—35)	3	1.3 ± 0.3 ^{c)}	30.9 ± 1.5 ^{d)}	73.8 ± 2.4 ^{d)}	28.7 ± 1.1 ^{d)}	28.4 ± 1.1 ^{d)}
+SP-C(7—35)	3	3.1 ± 0.9	39.4 ± 3.3	56.9 ± 3.5 ^{c)}	39.9 ± 1.9 ^{c)}	38.7 ± 1.2 ^{c)}
+SP-C(1—32)	3	2.2 ± 1.3	27.2 ± 1.3 ^{d)}	71.0 ± 1.3 ^{d)}	29.6 ± 1.1 ^{d)}	28.6 ± 1.2 ^{d)}
+SP-C(1—31)	3	2.8 ± 2.1	27.8 ± 2.0 ^{c)}	65.3 ± 2.6 ^{d)}	32.0 ± 2.7 ^{d)}	31.3 ± 2.7 ^{d)}
+SP-C(1—30)	3	3.8 ± 1.5	35.2 ± 2.1	61.4 ± 3.9 ^{d)}	42.1 ± 1.0	39.5 ± 2.1 ^{c)}
+SP-C(1—29)	3	4.0 ± 1.4	36.8 ± 1.0	59.1 ± 1.7 ^{d)}	41.8 ± 1.9	40.7 ± 0.8 ^{c)}
+SP-C(1—27)	3	3.9 ± 1.2	38.6 ± 2.2	60.4 ± 2.2 ^{d)}	41.6 ± 1.3	40.9 ± 1.1 ^{c)}
+SP-C(1—16)	3	3.6 ± 1.1	35.1 ± 1.5 ^{c)}	61.9 ± 3.8 ^{d)}	45.5 ± 1.6	45.3 ± 1.6
+SP-C(4—30)	3	6.8 ± 1.8	36.5 ± 1.2	43.1 ± 1.7	44.7 ± 2.1	43.0 ± 1.8
+SP-C(5—31)	5	1.5 ± 0.2 ^{c)}	35.8 ± 0.3 ^{c)}	66.9 ± 1.8 ^{d)}	30.2 ± 1.3 ^{d)}	28.9 ± 1.1 ^{d)}
+SP-C(6—32)	5	1.2 ± 0.6 ^{d)}	32.8 ± 1.7 ^{d)}	73.7 ± 1.3 ^{d)}	30.8 ± 1.6 ^{d)}	29.3 ± 1.5 ^{d)}
+SP-C(7—33)	3	3.9 ± 1.4	38.2 ± 0.7	48.9 ± 3.1	44.7 ± 1.1	43.7 ± 1.3
+SP-C(6—12)	2	9.7	49.8	40.4	46.5	45.0
+SP-C(13—32)	2	6.5	43.5	45.6	43.5	42.8
+SP-C(6—20)	2	8.5	43.5	43.6	43.3	42.7
+SP-C(21—32)	2	3.8	46.4	46.7	45.5	43.8
+SP-C(6—12)-SP-C(13—32)	2	7.5	40.7	44.2	42.2	41.0
+SP-C(6—20)-SP-C(21—32)	2	7.6	41.2	41.6	47.5	44.0

A ternary lipid mixture of DPPC/PG/PA (75:25:10, w/w), admixed with or without synthetic peptides (2% by weight of phospholipids) was applied to the air-water interface at 1.5 μg of phospholipids cm^2 surface area and allowed to spread spontaneously for 3 min. Initial spreading concentrations in the films corresponded to 8.2 $\text{\AA}^2$ per mole of phospholipids. The surface area was then cycled from an area of 54.0 to 21.6 cm^2 and dynamic surface tension was recorded as described in Materials and Methods. Relative change in surface area was 60% and cycling time was 2.4 min. *a)* Minimum surface tension. *b)* Maximum surface tension. *c)* $p < 0.01$, and *d)* $p < 0.001$ compared with the value of DPPC/PG/PA. Each value represents the mean $\pm$ S.D. ($n = 2-5$).

40–50% ($p < 0.01$ vs. native SP-C). Thus, SP-C (5–31) and SP-C (6–32) were the minimum peptides which showed excellent surface activity nearly identical with those of native SP-C. Though having the same number of amino acids as these peptides, SP-C (1–27), SP-C (4–30) and SP-C (7–33) were less effective than SP-C (5–31) or SP-C (6–32) in lowering the equilibrium surface tension during spreading and in lowering the compression area to yield a surface tension below 10 mN/m ($p < 0.01$). The peptide-lipid mixture containing a pair of two short peptide fragments corresponding to the partial sequences of SP-C (6–32), *e.g.*, SP-C (6–12) and SP-C (13–32) and SP-C (6–20) and SP-C (21–32), were also less active than that containing SP-C (5–31) or SP-C (6–32) alone.

Characterization of SP-C (6–32) To characterize further the biophysical activity of SP-C (6–32), its adsorption to the air-water interface, reproducibility of dynamic surface tension-area diagrams and physiological activity in the pulmonary mechanistics of premature rabbit neonates were analyzed.

The adsorption kinetics of each peptide-lipid mixture was evaluated by measurement of surface tension after injection of the sample underneath an air-water interface. Adsorption rates of each sample were very similar to their spreading rates. SP-C (6–32) and SP-C (1–35) were able to reproduce the properties of bovine native SP-C. Addition of these peptides induced a rapid adsorption of phospholipids at the air-water interface and led to a surface tension of 32.7–41.2 mN/m at 1–5 min after injection (Table 2). For a ternary lipid mixture of DPPC/PG/PA alone, the adsorption curve was much slower and

the equilibrated tensions (55.8–57.6 mN/m at 1–5 min) were significantly higher than those of lipid-peptide mixtures ($p < 0.01$ vs. SP-C (6–32)).

The relevance of the hysteresis in phospholipid films for lung surfactant function *in vivo* has been demonstrated. To investigate the contribution of the hydrophobic proteins to the hysteresis in the surface tension observed in the films, measurements were made of dynamic surface tension-area isotherm on the films of DPPC/PG/PA alone or DPPC/PG/PA plus hydrophobic protein compressed beyond the collapse point, and the extent of respreading of the films was determined using the collapse plateau ratio criterion. The films were dynamically cycled at 1 min/cycle for 7 consecutive cycles and the total area compression was always 60%. As shown in Table 3 and Fig. 3, addition of 2% (w/w) of SP-C (6–32) in DPPC/PG/PA led to a greater hysteresis in the surface tension-area isotherms than those found for films of DPPC/PG/PA or Exosurf®. In the first cycle, starting after spreading to 23 mN/m, the film had to be compressed by only 19.6% to reach a surface tension below 10 mN/m, and the length of the collapse plateau of the film of DPPC/PG/PA plus SP-C (6–32) was longer than that of the same film without SP-C (6–32). Upon expansion, the surface tension rose to a maximum of 23.6 mN/m. The hysteresis loop was very reproducible from the second cycle and continued to be relatively stable up through the 7 cycles of dynamic compression-expansion. Values of the ratios for cycle 2/cycle 1, cycle 4/cycle 1 and cycle 7/cycle 1 are shown in Table 3. The collapse plateau length ratios for the cycling of films showed the superior respreading of the films

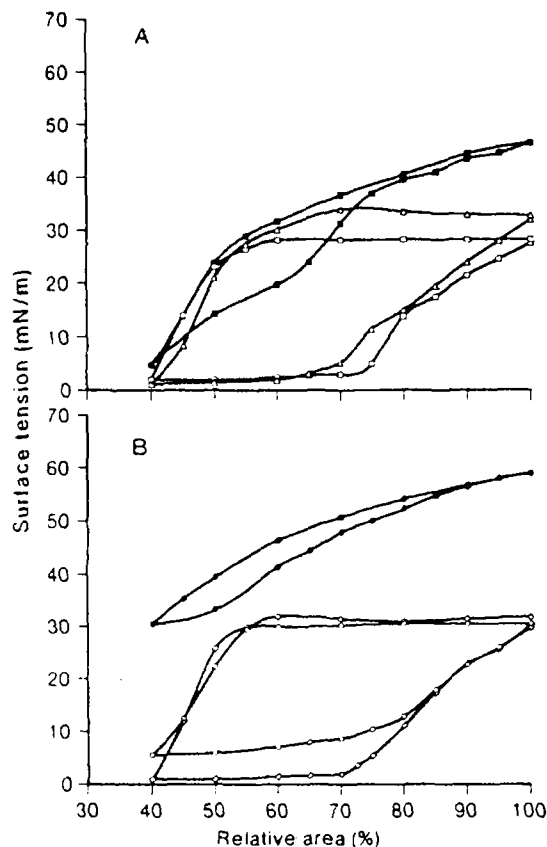


Fig. 2. Dynamic Compression-Expansion Cycles of Surfactant Preparations

Details as in Table 1. Typical isotherms of cycle 4 are shown. A, DPPC:PG:PA and DPPC:PG:PA plus SP-C (1-35) (□) and SP-C (6-32) (Δ) at a concentration of 2% (w/w) relative to the lipids; B, Surfacten® (○), Exosurf® (●) and DPPC:PG:PA plus bovine native SP-C (◇) at a concentration of 2% (w/w) relative to the lipids.

Table 2. Comparison of Surface Adsorption Characteristics of DPPC:PG:PA Plus SP-C Related Peptide, Surfacten®, and Exosurf®

Sample	Surface adsorption ^a		
	at 1 min (mN/m)	at 3 min (mN/m)	at 5 min (mN/m)
DPPC:PG:PA	57.6 ± 5.1	56.7 ± 4.7	55.8 ± 4.3
+ SP-C(1-35)	35.0 ± 3.1 ^a	33.9 ± 3.2 ^a	32.7 ± 2.9 ^a
+ SP-C(6-32)	41.2 ± 3.8 ^{ab}	38.0 ± 3.6 ^a	36.3 ± 3.3 ^a
+ Bovine SP-C	36.9 ± 2.8 ^a	35.3 ± 2.1 ^a	34.9 ± 1.6 ^a
Surfacten®	38.0 ± 2.2 ^{ab}	36.2 ± 1.8 ^a	34.7 ± 1.2 ^b
Exosurf®	57.5 ± 0.2	56.7 ± 0.2	55.8 ± 0.3

A surfactant preparation containing 1.5 mg of phospholipids was injected into the subphase of 30-ml saline in a round Teflon trough (5 cm in diameter and 2 cm deep) with stirring at a rate of 60 rpm. The change in surface tension was then measured at indicated times after stirring stopped. The final adsorption concentrations in the films corresponded to 0.16 Å² per mole of phospholipids. at *p* < 0.01 and *b* *p* < 0.001 compared with the value of DPPC:PG:PA. Each value represents the mean ± S.D. (*n* = 3).

containing hydrophobic protein over those of DPPC/PG/PA alone. The film containing SP-C (6-32) gave the cycle 2/cycle 1, cycle 4/cycle 1 and cycle 7/cycle 1 ratios of 0.94, 0.83 and 0.53, respectively, while the DPPC/PG/PA film gave corresponding ratios of 0.86, 0.14 and 0.13, showing that SP-C (6-32) increased the respreading of peptide-lipid film about 4-6 times over that of the lipid film at

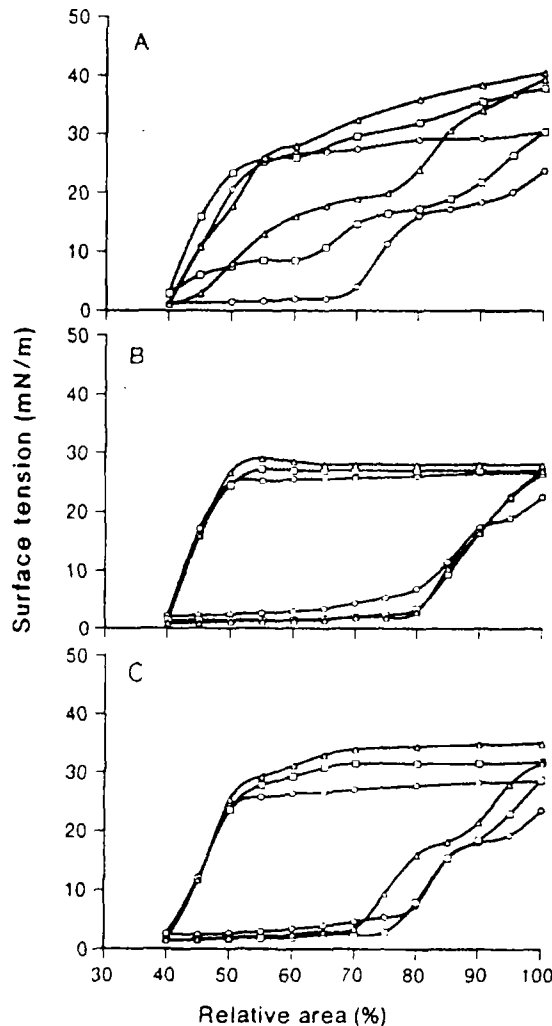


Fig. 3. Dynamic Compression-Expansion Behavior of DPPC:PG:PA (A) and DPPC:PG:PA Plus SP-C (1-35) (B) and SP-C (6-32) (C)

Surface films spread from a solvent of hexane:ethanol (9:1, v/v) to 13 Å² per molecule of phospholipids were continuously compressed and expanded at a rate of 2.4 min/cycle. Relative change in surface area was 60%. Typical isotherms of cycles 1 (○), 2 (□) and 4 (Δ) are shown.

the fourth and the seventh cycles. The seventh respreading of the film containing SP-C (6-32) was slightly lower than the second and the fourth respreading. The effect of SP-C (6-32) on respreading of DPPC/PG:PA was similar to those of chemically synthesized SP-C (1-35) and bovine native SP-C.

Immature newborn rabbits, delivered at a gestational age of 27 d, are commonly used for experimental evaluation of surfactant substitutes. *In situ* quasi-static pressure-volume properties of lungs of prematurely delivered rabbit neonates after tracheal instillation of the ternary lipid mixture in the presence and the absence of hydrophobic peptide are shown in Fig. 4. For comparison, the data are also given from littermate controls and from neonates at term (gestational age of 30 d), which received no surfactant. In a surfactant-deficient immature rabbit fetus with a gestational age of 27 d, quasi-static lung volumes at 5 cm H₂O deflation pressure were 2.8 ± 1.8 ml/kg body weight. At a dose of 50 mg phospholipids/kg, tracheal instillation of DPPC:PG:PA plus SP-C (6-32)

Table 3. Comparison of Respreading Characteristics of DPPC PG PA Plus SP-C Related Peptide, Surfactant[®], and Exosurf[®]

Sample	Surface area at 10 mN m			Collapse plateau length ratio		
	Cycle 2 (%)	Cycle 4 (%)	Cycle 7 (%)	2:1	4:1	7:1
DPPC PG PA	61.8 ± 4.3	49.8 ± 4.8	49.3 ± 4.7	0.86 ± 0.02	0.14 ± 0.01	0.13 ± 0.01
+ SP-C(1-35)	81.0 ± 3.2 ^a	80.1 ± 4.0 ^a	79.4 ± 2.9 ^a	0.99 ± 0.02 ^a	1.00 ± 0.02 ^a	0.97 ± 0.04 ^a
+ SP-C(6-32)	80.4 ± 2.7 ^a	74.7 ± 3.3 ^a	66.3 ± 3.2 ^a	0.94 ± 0.01 ^a	0.83 ± 0.04 ^a	0.53 ± 0.04 ^a
+ Bovine SP-C	73.5 ± 3.2 ^a	76.7 ± 2.2 ^a	75.8 ± 1.4 ^a	0.93 ± 0.02 ^a	1.01 ± 0.01 ^a	1.01 ± 0.04 ^a
Surfactant [®]	78.7 ± 1.5 ^a	77.3 ± 2.3 ^a	76.2 ± 1.5 ^a	0.96 ± 0.02 ^a	0.96 ± 0.03 ^a	0.94 ± 0.01 ^a
Exosurf [®]	< 40	< 40	< 40	0.44 ± 0.02 ^a	0.26 ± 0.03 ^a	0.12 ± 0.01

A sample was spread from a solvent of hexane:ethanol (9:1, v/v) on the air-water interface at $13 \text{ \AA}^2$ per molecule of phospholipids. The surface area was then cycled from an area of 54.0 to 21.6 cm^2 and dynamic surface tension was recorded as described in Materials and Methods. Relative change in surface area was 60%, and cycling time was 2.4 min. (a) $p < 0.01$ and (b) $p < 0.001$ compared with the value of DPPC PG PA. Each value represents the mean $\pm$ S.D. ($n = 3$).

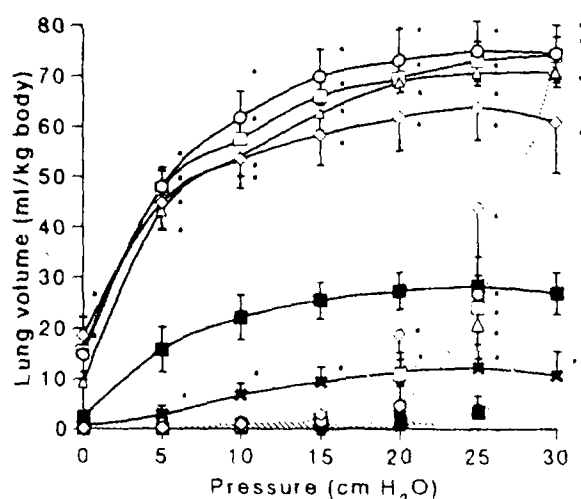


Fig. 4. Pressure-Volume Characteristics of Premature Rabbit Neonates after Tracheal Instillation of Surfactant[®] (○), DPPC PG PA (■) and DPPC PG PA Plus SP-C (1-35) (□) and SP-C (6-32) (△).

Rabbit neonates at a gestational age of 27 d were treated with surfactant preparations at a dose of 50 mg of phospholipids/kg body weight or with saline (×). For comparison, the data from neonates at term (◇) receiving no surfactant preparation are also shown. Solid lines, measurements during deflation; dashed lines, measurements during inflation. The data represents the mean $\pm$ S.D. ($n = 4-10$). * Significantly different from DPPC PG PA, $p < 0.001$.

improved the quasi-static pressure-volume properties of immature rabbits in which a lung volume of 43.1 ± 3.5 ml/kg at 5 cm H_2O deflation pressure was comparable with those observed in immature rabbits receiving other peptide-lipid surfactants and with that of the neonates at term (44.8 ± 5.5 ml/kg). Furthermore, the pressure-volume characteristics of these peptide-lipid mixtures were statistically better than those of the ternary lipid mixture ($p < 0.01$, lung volume of 15.8 ml/kg at 5 cm H_2O deflation pressure).

DISCUSSION

In these studies, a number of truncated peptides having decreasing lengths of native SP-C sequence were synthesized to define the minimum segment(s) required to restore the biophysical activity of native SP-C. This research led to the discovery that SP-C related peptides having a core sequence of 27 residues, i.e., (C)CPVHLKRLIVVVVVVLIVVVIVGAL(L), could fully enhance the abilities of phospholipids to rapidly reduce surface

tension of the air-water interface *in vitro* when mixed with the lipid mixture of DPPC PG PA (75:25:10, w/w) (Table 2).

As shown in Fig. 3, the 2nd and 7th hysteresis loops of DPPC PG PA alone demonstrated that the length of the collapse plateau was considerably shorter than that of the 1st cycle and these maximum surface tensions past collapse were remarkably higher than the initial surface tension of the film. This indicated that some fractions of DPPC molecules may be lost from the air-water interface by the formation of local, irreversible collapse structures or by incorporation into subphase lamellae during film collapse.^{29,31,32} The lengths of squeeze out plateau of PG and PA just below 20 mN m remained stable during dynamic cycles of DPPC PG PA film. The addition of SP-C (6-32), as well as native SP-C or SP-C (1-35), led to improved respreadability of DPPC PG PA. The second and fourth cycles of peptide-lipid film displayed greater and more reproducible hysteresis than did those for the lipid film. These results indicate that integration of SP-C into lipid film leads to reversible collapse structures of peptide-lipid film,³³ of which almost all lipid material spread easily to retain low maximum surface tensions and to keep large collapse plateaus of hysteresis loops during successive dynamic cycles.

An amphipathic structure with long hydrophobic α -helix may be important for these functions of native SP-C and SP-C related synthetic peptides as discussed below.

SP-C (6-32), one of the minimum peptides for good surface properties in a Langmuir-Wilhelmy balance and fetal rabbit assays as stated above, is also an extremely hydrophobic, amphipathic peptide consisting of the core sequence of CPVHLKRLIVVVVVVLIVVVIVGALL which can be divided into two specific regions, i.e., a hydrophilic region (residues 6-12 of SP-C) and a hydrophobic region (residues 13-32 of SP-C). Even though peptides have a longer stretch of native sequence including the entire length of one of these specific regions, any deletion of amino acid residue(s) in the other region significantly reduced their activity. On the basis of the NMR solution structure of human SP-C described by Johansson *et al.*,^{34,35} the hydrophobic region of residues 13-32 together with a partial sequence of the hydrophilic region of residues 9-12 including two conserved basic amino acids, Lys-11 and Arg-12, allows formation of an α -helical structure. The length of the hydrophobic part of

the α -helix in SP-C (5–31) and SP-C (6–32) is 27–29 Å, which is closer to the hydrophobic thickness of 26.3 Å in the liquid-crystalline phase of the DPPC bilayer than to that in the gel phase (39.4 Å).^{36–38} In contrast, the peptides having shorter hydrophobic regions, such as SP-C (1–30) of which the hydrophobic part of α -helix is less than 26 Å long, was demonstrated to be significantly less active than native SP-C. These results strongly reinforce the idea that the extremely hydrophobic α -helix of SP-C exactly spans the hydrophobic part of a fluid DPPC bilayer.^{37–39} Furthermore, our present data clearly define the minimum length of the hydrophobic α -helical structure of native SP-C required for sufficient function to improve surface activity of phospholipids.

In agreement with previous reports,^{21,23,24} the ability of SP-C to enhance the surface activity of phospholipids was not affected by the absence of two palmitoyl groups which are linked to Cys-5 and Cys-6 in native SP-C. Johansson *et al.* described that an analogue of SP-C in which two cysteines were replaced by serines also maintained activity.²³ We undertook an experiment of this and confirmed that the analogue of SP-C (6–32) significantly improved the surface activity of the lipid mixture (data not shown). Therefore, it is assumed that Cys-6 of SP-C (6–32) is not essential to the activity of this peptide. Deletion of the first 5 residues in the N-terminal segment did not cause much loss of activity of SP-C related synthetic peptides having the entire length of the C-terminal α -helical structure, but a remarkable activity loss was observed when further amino acids were removed from the N-terminus of SP-C. The N-terminal hydrophilic segment of SP-C (6–32), CPVHLKR, contains the evolutionarily conserved cysteine and two basic amino acid residues. As discussed above, the last 4 residues of this segment locate at the N-terminal end of the well-defined α -helical structure of SP-C encompassing residues 9–34. It has been shown that the conserved basic amino acid residues, Lys-11 and Arg-12, are suitably located for interacting with the phosphatidylcholine moieties of DPPC.⁴⁰ Taken together, these observations suggest that the N-terminal hydrophilic segment of SP-C (6–32) may be essential to maintain the stable structure of α -helix and to anchor the SP-C (6–32) molecules to the DPPC bilayer by specific interaction between positively charged amino groups of peptides and negatively charged head groups of phosphatidylcholine moiety.

Consequently, this study identified the minimum amphipathic sequences of SP-C having the most activity of native SP-C. Although many questions concerning the secondary structure of these SP-C related peptides in their interaction with phospholipids remain to be solved, these results are promising for the development of new artificial surfactant preparations based on synthetic analogues of SP-C.

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Synthetic peptide-containing surfactants

Evaluation of transmembrane versus amphipathic helices and surfactant protein C poly-valyl to poly-leucyl substitution

Gunhild NILSSON¹, Magnus GUSTAFSSON², Guy VANDENBUSSCHE³, Edwin VELDHUIZEN⁴, William J. GRIFFITHS², Jan SJÖVALL², Henk P. HAAGSMAN⁴, Jean-Marie RUYSSCHAERT³, Bengt ROBERTSON⁵, Tore CURSTEDT¹ and Jan JOHANSSON²

¹ Department of Clinical Chemistry, Karolinska Institutet at Karolinska and Danderyd Hospital, Stockholm, Sweden

² Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden

³ Laboratoire de Chimie-Physique des Macromolécules aux Interfaces, Université Libre de Bruxelles, Brussels, Belgium

⁴ Laboratory of Veterinary Biochemistry, Utrecht University, Utrecht, The Netherlands

⁵ Research Unit for Experimental Perinatal Pathology, Karolinska Hospital, Stockholm, Sweden

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Pulmonary surfactant contains two hydrophobic proteins, SP-B and SP-C. With the aim of identifying synthetic SP-B and SP-C substitutes for replacement therapy of respiratory distress syndromes, we have studied two transmembrane peptides and two amphipathic peptides that are located in the plane of a phospholipid bilayer. One amphipathic peptide was designed by changing the amino acid sequence, but not the composition or size, of the 21-residue peptide KL₄. This peptide, designated KL_{2,3}, from its spacing of nonpolar and polar residues, exhibited similar α -helical content as KL₄ but was oriented along a phospholipid bilayer plane, in contrast to the transmembrane orientation of KL₄ in the same environment. The second amphipathic peptide analyzed was succinyl-LLEKLLEWLK-amide (WMAPI0). KL₄ more efficiently accelerated the spreading of a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (Pam₂-GroPCho)/phosphatidylglycerol (PtdGro)/palmitic acid (PamOH), 68:22:9 (by mass), at an air/water interface than did any of the amphipathic peptides. Similarly, KL₄, but not KL_{2,3}, when present in an interfacial monolayer composed of Pam₂-GroPCho/1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol, 7:3 (by mass), increased lipid insertion from subphase vesicles.

An SP-C analogue, SP-C(Leu), with all helical valyl residues in native SP-C replaced with Leu and the palmitoylcysteines at positions 5 and 6 replaced with Ser, but otherwise with essentially the same primary structure as the native peptide, was analyzed. SP-C(Leu) exhibited similar α -helical content as native SP-C and a transmembrane orientation and, in contrast to poly-valyl-containing synthetic peptides, it folds into a helical conformation after acid-induced denaturation. SP-C(Leu) accelerated the spreading of Pam₂-GroPCho/PtdGro/PamOH, 68:22:9 (by mass), almost identically to native SP-C, and lowered the surface tension during rapid cyclic film compressions in a pulsating bubble surfactometer to near zero and 43 mN/m at minimum and maximum bubble size, respectively. Airway instillation of 2% (by mass) SP-C(Leu) combined with Pam₂-GroPCho/PtdGro/PamOH in preterm rabbit fetuses improved dynamic lung compliance by about 30% compared with untreated controls.

Keywords: pulmonary surfactant; surfactant protein B; surfactant protein C; transmembrane peptide; amphipathic peptide.

Correspondence to J. Johansson, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, S-171 77 Stockholm, Sweden
Phone: +46 8 7287781.

Fax: +46 8 337462.

E-mail: jan.johansson@mbb.ki.se

URL: <http://www.mbb.ki.se/>

Abbreviations. ATR-FTIR, attenuated total reflection-Fourier transform infrared; DodPCho, dodecylphosphocholine; EBE, electric-magnetic-electric; ESI, electrospray ionization; MALDI-TOF, matrix-assisted laser desorption and ionization time-of-flight; MLV, multilamellar vesicles; Myr₂GroPCho, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; Ole₂GroPCho, 1,2-di-oleoyl-*sn*-glycero-3-phosphoglycerol; PamCys, palmitoylcysteine; Pam₂GroPCho, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; PamOH, palmitic acid; PamOleGroPCho, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PamOleGroPCho, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; PtdGro, phosphatidylglycerol; RDS, respiratory distress syndrome; SP, surfactant protein; SUV, small unilamellar vesicles; TOFFPD, time-of-flight focal plane detector.

Pulmonary surfactant is formed by the alveolar epithelium, and is essential for normal lung function. Exogenous surfactant preparations, obtained mainly from animal lungs, are currently used for treatment of respiratory distress syndrome (RDS) in preterm infants. The dominating components of surfactant are phospholipids, whereof 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (Pam₂GroPCho) is the main species, and proteins. The water-soluble surfactant proteins A and D (SP-A and SP-D) are C-type lectins that most likely are primarily involved in pulmonary host-defence functions, although both proteins are also capable of interacting with lipids. In accord with this, knockout of the SP-A gene in mice produces no apparent respiratory dysfunction [1]. In contrast, several lines of evidence indicate that the hydrophobic SP-B and SP-C are of significant importance for respiratory function. Knockout of the SP-B gene in mice produces a lethal phenotype that exhibits respiratory distress from birth [2] and blocking of SP-B with a monoclonal antibody

induces respiratory failure in newborn experimental animals [3]. Likewise, genetic deficiency of proSP-B in humans causes severe respiratory dysfunction in early infancy [4]. In both hereditary SP-B deficiency and in SP-B knockout mice the proteolytic generation of SP-C from proSP-C is interfered with, which causes accumulation of an 8–12-kDa proform instead of the 4.2-kDa SP-C peptide. Furthermore, experience from replacement therapy of RDS in premature infants indicates that SP-B and SP-C are essential constituents of exogenous surfactants [5]. However, the supply of natural surfactant is limited, the production procedure is time-consuming and the proteins from animal sources may be immunogenic, all suggesting that an artificial surfactant based on synthetic components would be of value.

SP-B and SP-C have been extensively studied in terms of structural properties [6]. SP-B is a 17.4-kDa homodimer, composed of two 79-residue polypeptides that each contain three intrachain disulphide bridges. The secondary structure of SP-B is dominated by α -helices and, based on structural data and amino acid sequence comparisons of SP-B and other saposins, a four helix topology of these polypeptides has been proposed [7]. The four helices are amphipathic, i.e. their circumferences exhibit one nonpolar and one polar face, and SP-B probably lacks transmembrane helices. The recently determined NMR structure of a saposin domain confirmed the presence of these helices but revealed that in the monomeric water-soluble saposin protein studied, the nonpolar parts of the amphipathic helices constitute a hydrophobic protein core which is not exposed to the solvent [8]. This structure does not explain how the hydrophobic and dimeric SP-B interacts with phospholipid bilayers [6, 7, 9]. SP-C is a 4.2-kDa lipopeptide. It contains a 37-Å-long α -helix, encompassing residues 9–34, with a 26-Å-long very hydrophobic poly-valyl part [10]. In addition, one (canine) or two (all other species analyzed) palmitoyl group(s) are thioester-linked to Cys5 (in canine SP-C) and Cys6 [11, 12]. The SP-C α -helix is oriented in a transmembrane way in a fluid phospholipid bilayer [6] where the poly-valyl helical part may interact optimally with the fatty acyl-chains [13]. In a Pam₂GroPCho monolayer, the SP-C helix makes an approximately 70° tilt relative to the interface normal, maximising the interactions with the lipid acyl chains also in this environment [14]. The membrane-spanning helix is needed for surfactant activity [15] and neutralization of the positive charges of SP-C by chemical modification causes a significant impairment of its functional properties [16]. Synthetic replicas of SP-C are prone to form insoluble aggregates with the peptides in extended conformations, both in phospholipid micelles and in organic solvents. In contrast, a synthetic version of a transmembrane segment from bacteriorhodopsin that lacks a poly-valyl part forms a helix under the same conditions [17]. This implies that the poly-valyl segment of SP-C does not readily fold into a helical conformation in synthetic analogues. Furthermore, non-helical SP-C was found to exhibit inferior biophysical surfactant activity compared with helical SP-C [17]. It has, however, recently been reported that synthetic SP-C-based peptides containing poly-valyl segments and combined with lipids exhibit excellent biophysical activity, and that such mixtures improved static lung compliance of immature rabbits to levels similar to those of mature neonates [18]. The secondary structures of these peptides were not reported [18].

Artificial surfactants have been produced by mixing synthetic lipids and polypeptides corresponding to native SP-B or SP-C, or parts thereof, or other simplified peptide structures [17–23]. Many of these peptide-containing surfactant preparations are superior to phospholipids alone but their biophysical and biological activities are in most cases inferior to natural pulmonary surfactant. To enable clear-cut comparisons two transmembrane and two amphipathic peptides have now been synthe-

sized and analyzed with respect to their structures and orientations in phospholipid bilayers, as well as to their effects on the surface activity of different lipid mixtures.

EXPERIMENTAL PROCEDURES

Lipids. Pam₂GroPCho, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PamOleGroPCho), 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (Ole₂GroPGro), 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphoglycerol (PamOleGroPGro), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (Myr₂GroPCho), egg phosphatidylglycerol (PtdGro) and palmitic acid (PamOH) were purchased from Sigma or Avanti Polar lipids. The lipids were used without further purification since purity checks by thin-layer chromatography showed single spots.

Peptide synthesis, purification and characterization. Peptides were synthesized by solid-phase methodology and *tert*-butyloxycarbonyl chemistry in an Applied Biosystems 430A instrument. KL₄ [22], KL_{2,3} and SP-C(Leu) contain unmodified N-terminus and C-terminus (Fig. 1). WMAP10 [21, 23] was synthesized with a C-terminal amide, and before removal of side-chain protecting groups its N-terminal amino group was succinylated by incubation with succinic anhydride at room temperature for 30 min in dimethylformamide/dichloromethane/triethylamine, 10:10:1 (by vol.). All side-chain-protecting groups (except a Trp-protecting formyl group, see below) were removed and the peptides liberated from the synthesis resin by cleavage with anhydrous hydrogen fluoride/anisole/dimethylsulphide, 10:1:1 (by vol.). After extraction of protecting groups and scavengers with diethyl ether, the hydrophobic peptides KL₄, KL_{2,3} and SP-C(Leu) were extracted from the resin with dichloromethane/trifluoroacetic acid, 3:1 (by vol.), while the hydrophilic peptide WMAP10 was extracted using 30% aqueous acetic acid. The protecting formyl group of the WMAP10 Trp8 imino group was removed by incubation of the peptide (5 mg/ml) in 0.4 M ethanolamine/6 M guanidine/HCl, pH 11, at 0°C for 5 min, whereafter the reaction was stopped by the addition of HCl until the pH reached 6.0. The deformylation reaction was followed by monitoring the increase in absorbance at 280 nm relative to the absorbance at 300 nm.

WMAP10 was purified from the ethanolamine/guanidiniumHCl solution obtained after deformylation by reverse-phase HPLC on a C₁₈ column (2.2 cm×25 cm, Vydac) using a linear gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid and a flow of 10 ml/min. This yielded one major peak that was confirmed to correspond to the expected product by electrospray ionization (ESI) mass spectrometry.

Because of their hydrophobic nature KL₄, KL_{2,3} and SP-C(Leu), after evaporation of the solvents used for extraction from the resin, were solubilized in ethanol and purified by reverse-phase HPLC using a C₁₈ column and a linear gradient of 2-propanol/0.1% trifluoroacetic acid in 60% methanol/0.1% trifluoroacetic acid or 75% ethanol/0.1% trifluoroacetic acid [24]. Relevant peaks were collected and the peptides identified by amino acid analysis, matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) mass spectrometry, and, in the case of SP-C(Leu), also by amino acid sequence analysis and ESI mass spectrometry.

Native SP-C was isolated from pig pulmonary surfactant phospholipids by chromatography on Sephadex LH-60 in chloroform/methanol/0.1 M HCl, 47.5:47.5:5 (by vol.) [25], and quantified by amino acid analysis.

ESI mass spectrometry was performed on an AutoSpec time-of-flight focal plane detector (TOFFPD) hybrid tandem mass spectrometer. This instrument, described in detail elsewhere [26,

The figure displays four circular diagrams, each representing a different protein structure. Each diagram consists of a circle with internal lines forming a star-like pattern and external labels (K, L, G, W, M, A, E, R) indicating specific residues or domains.

- KL₄**: A circular diagram with labels K1, K16, K6, K21, and K11. The internal lines form a complex star pattern.
- KL_{2.3}**: A circular diagram with labels K4, K11, K18, K7, K14, and L21. The internal lines form a complex star pattern.
- WMAP10**: A circular diagram with labels G, W, L1, L, E7, E3, and K10. The internal lines form a complex star pattern.
- SP-C(Leu)**: A circular diagram with labels G, M, L, V8, A, G3, L, I, L, K10, and L. The internal lines form a complex star pattern.

27], consists of a double-focusing arrangement of electric-magnetic-electric (EBE) sectors as mass spectrometer 1 (MS1) and an orthogonal time-of-flight mass analyser as MS2. Samples (16 pmol/ μ l) were introduced into the electrospray ion source either by continuous infusion or loop injection at a flow rate of 3 μ l/min. The solvents employed were a 1:1 (by vol.) mixture of chloroform and methanol containing 2% acetic acid for the analysis of SP-C(Leu) and a 50% aqueous solution of methanol containing 1% acetic acid for the analysis of WMAP10. The ion source was operated at a potential of 4 kV in the positive ion mode. For the recording of conventional mass spectra the instrument was tuned to a resolution of approximately 2000 (10% valley) and the magnet scanned at a rate of 15 s/decade over an m/z range between 100 and 5000. By altering the cone to skimmer voltage differential it was possible to alter the population of ions in a given charge state. Higher resolution (5000, 10% valley) spectra were also recorded over mass ranges just sufficient to bracket SP-C(Leu) peaks between calibrant ion peaks.

Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy. Before preparation of the samples for infrared spectroscopy, the trifluoroacetate counterions from the purification procedure were removed by twice lyophilizing the peptides in presence of 10 mM hydrochloric acid [28]. KL_{2,3} and KL₄ in ethanol or SP-C(Leu) in chloroform/methanol 1:1 (by vol.) were added to Pam₃GroPCho/PtdGro/PamOH (68:22:9, by mass) in chloroform/methanol (98:2, by vol.) in the polypeptide/lipid proportion 1:10 (by mass). The solvents were evaporated under nitrogen and solvent traces were removed by keeping the residue under reduced pressure overnight. The

polypeptide/lipid films were hydrated in 2 mM Hepes, pH 7.4, at a final lipid concentration of 1 mg/ml. The hydrated films were incubated 15 min at 45°C and the peptide/lipid mixtures were resuspended by vortexing in presence of small glass beads. The WMAP10 peptide was reconstituted in a lipid matrix by addition of the peptide in 2 mM Hepes, pH 7.4, to a Pam₂-GroPCho/PtdGro/PamOH (68:22:9, by mass) film and resuspended as described above. Peptide-containing lipid vesicles were isolated by centrifugation in a continuous sucrose gradient (2% to 30%) at 100 000 g for 16 h at 4°C. The location of phospholipids in the gradient was identified by a colorimetric test for choline and peptide-containing fractions were pooled and dialysed extensively against 2 mM Hepes, pH 7.4.

ATR-FTIR spectra were recorded at room temperature on a Bruker IFS-55 spectrometer, equipped with a liquid nitrogen cooled mercury cadmium telluride detector, in the range from 900 cm⁻¹ to 4000 cm⁻¹ with a nominal resolution of 2 cm⁻¹. The internal reflection element was a 50×20×2 mm germanium crystal with an aperture angle of 45°, yielding 25 internal reflections. Each spectrum was recorded as an average of 1024 scans. Films of peptides and peptides associated with Pam₂-GroPCho/PtdGro/PamOH (68:22:9, by mass) were obtained by slowly evaporating the sample on the germanium plate under nitrogen at room temperature.

Hydrogen to deuterium (H/D) exchange of readily accessible amide protons were obtained by flushing the sample on the ATR plate sealed in a sample holder with D₂O-saturated N₂. The secondary structure of the peptides was quantified from the amide I band, after H/D exchange for one hour, using a Fourier self-deconvolution procedure, for details see [29–31].

To determine the orientation of peptide α -helices and lipid acyl chains, additional spectra were recorded with parallel (/ /) and perpendicular ($\perp$) polarized light with respect to the incident plane. The mean angle between the helix axis and a normal to the ATR plate was calculated from the dichroic ratio $R_{ATR} = A'_{\parallel}/A'_{\perp}$. The γ_{CH_2} transition at 1200 cm⁻¹, the transition dipole moment of which lies parallel to the all-*trans* hydrocarbon chains, was used to characterize the lipid acyl chain orientation, see [30].

Circular dichroism (CD) spectroscopy. For analysis of peptide secondary structure in phospholipid micelles, peptides were solubilized in 20 mM dodecylphosphocholine (DodPCho; critical micelle concentration $\approx$ 1 mM) in 50 mM sodium phosphate, pH 6.0, and diluted to 10 mM DodPCho in the same buffer. CD spectra between 184 nm and 260 nm were recorded at room temperature with a Jasco-720 instrument using a scan speed of 20 nm/min, and a resolution of 2 data points/nm. The residual molar ellipticity was calculated after determination of peptide concentration by amino acid analysis. Content of helical structure was estimated from the molar ellipticities at 208 nm and 222 nm [17].

Preparation of peptide/lipid mixtures and lipid vesicles. Lipids, dissolved in chloroform/methanol, 98:2 (by vol.), were mixed in the mass proportions Pam₂-GroPCho/PamOleGroPCho/Ole₂-GroPGro 55:35:10 [25], Pam₂-GroPCho/PtdGro 7:3 [31–33] or Pam₂-GroPCho:PtdGro:PamOH 68:22:9 [34]. The lipid solution was added to native porcine SP-C in chloroform/methanol, 1:1 (by vol.), or to synthetic polypeptides in the proportions polypeptide/lipid 0–5:100 (by mass). The solvents were evaporated under nitrogen and the residue was suspended in 150 mM NaCl by sonication (50 W, 48 kHz), giving a lipid concentration of 10 mg/ml. For the monolayer experiments multilamellar vesicles (MLV) were prepared by dissolving Pam₂-GroPCho/PamOleGroPGro, 9:1 (by mass), in chloroform/methanol, 1:1 (by vol.), drying the mixture under a stream of nitrogen at room temperature, and hydrating the lipid film in 25 mM Hepes,

pH 7.0, by vortexing for 1 min at 55–60°C. Small unilamellar vesicles (SUV) with an approximate size distribution of 60–100 nm were prepared by sonicating MLV for 5×20 s with 12-s intervals at 30 W and 55°C using a Branson B12 sonifier equipped with a 0.5 in flat-top disrupter tip. The resulting SUV were kept at 37°C and used on the same day as they were prepared.

The effects of WMAP10 on the turbidity of lipid vesicles was measured by adding the peptide (1 mg/ml) in 50 mM sodium phosphate, pH 7.2, to a 1 mg/ml solution of Myr₂-GroPCho in the same buffer, to a final lipid/peptide mass ratio of 10:1. The mixture was incubated at 29°C for 30 min and the absorbance at 540 nm was monitored.

Surface spreading and monolayer studies. Kinetics of film formation for various peptide/lipid preparations at an air/liquid interface were measured at 32°C with a Wilhelmy balance equipped with a Teflon trough (18 cm²), containing a 20 ml hypophase of 150 mM NaCl. The surfactant preparation (0.5 mg or 1 mg of lipids) was added as a droplet falling onto the hypophase from a level of about 1 cm. The distance between the site of application and the Wilhelmy plate was similar in all experiments, about 4 cm. Surface pressure was recorded continuously during 1 min. With this method, the rate of film formation reflects a combination of bulk surfactant flow along a surface tension gradient, and adsorption of surfactant molecules at the air/liquid interface. However, in the following, we will refer to this complex phenomena simply as spreading. The presented values are the mean of three different recordings.

Monolayer experiments were performed at 37°C in a temperature-controlled box by the Wilhelmy plate method, using a Cahn 2000 electrobalance. A 5.5-ml Teflon trough was filled with subphase (25 mM Hepes, pH 7.0) and stirred continuously. Lipids (Pam₂-GroPCho/PamOleGroPGro, 7:3 by mass) and KL₄ or KL_{2,3} mixed with lipids (lipid-to-peptide mass ratio 10:1 or 10:3) were dissolved in chloroform/methanol, 1:1 (by vol.). The mixture was spread on the subphase to a surface pressure of 20 mN/m. SUV (40 μ M) made of Pam₂-GroPCho/PamOleGroPGro as described above were injected through an injection hole into the subphase, and lipid insertion from SUV was initiated by subsequent injection of CaCl₂ (final concentration 1.5 mM). The surface pressure increase as a function of time was recorded for 30 min.

Dynamic surface tension measurements. The dynamic surface properties of lipid/peptide mixtures showing rapid spreading in the surface balance systems described above were further evaluated with a pulsating bubble surfactometer. For these experiments, the mixtures were suspended in saline at a concentration of 10 mg/ml, and dynamic surface tension was recorded at 37°C during 50% cyclic compression of the bubble surface at a rate of 40 cycles/min. Values for surface tension at minimum and maximum bubble size ($\gamma_{\min}$, $\gamma_{\max}$) were recorded during 5 min of pulsation.

In vivo surfactant activity. The lipid/peptide mixture that exhibited the most promising *in vitro* surface properties was further tested in experiments on ventilated preterm newborn rabbits, obtained by hysterotomy at a gestational age of 27 days [35]. The animals were tracheotomised at birth and received, *via* the tracheal cannula, 2.5 ml/kg of the lipid/peptide mixture suspended in saline at a concentration of 20 mg/ml (higher concentrations could not be studied, for reasons explained below). Littermates receiving no material into the airways served as controls. The animals were kept in plethysmograph boxes at 37°C and were ventilated for 60 min with a tidal volume of approximately 10 ml/kg, a respiratory frequency of 40 breaths/min, and 50% inspiration time. Animals showing normal electrocardiograms (ECG) throughout the period of observation were counted

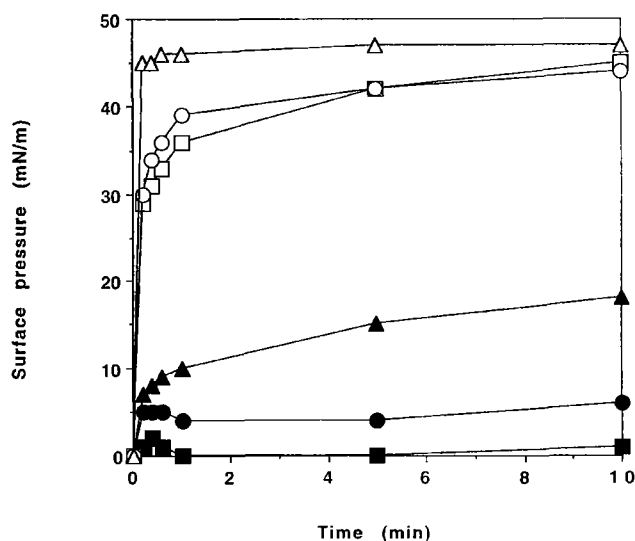


Fig. 2. Effects of palmitic acid and SP-C on surface spreading. Surface spreading and surface pressures achieved with the pure lipid mixtures (filled symbols) Pam₂GroPCho/PamOleGroPCho/Ole₂GroPGro 55:35:10 (by mass) (squares), Pam₂GroPCho/PtdGro 7:3 (by mass) (circles) and Pam₂GroPCho/PtdGro/PamOH 68:22:9 (by mass) (triangles) or with 5% native porcine SP-C added to the same lipid mixtures (open symbols). The samples were added as droplets onto the hypophase, 4 cm from the platinum plate, and surface pressure was determined versus time. The data are mean values of three separate experiments.

as survivors. Lung-thorax compliance was defined as the quotient between tidal volume (in ml/kg) and peak insufflation pressure (in cm H₂O). Heart blood was sampled from all animals at the end of the experiment for determination of pCO₂.

RESULTS

Influence of lipid composition on surface spreading. The pure phospholipid mixtures showed very low surface pressures, but the surface activity was augmented by addition of PamOH (Fig. 2). Addition of 5% native SP-C significantly increased the biophysical activity for all phospholipid mixtures (Fig. 2). When native porcine SP-C was mixed with Pam₂GroPCho/PtdGro/PamOH, 68:22:9 (by mass), the spreading and surface pressure were similar to those obtained by natural surfactant preparations [36]. Since the highest spreading rate and surface pressure were obtained with Pam₂GroPCho/PtdGro/PamOH, 68:22:9 (by mass), this lipid mixture was predominantly used in the further studies.

The transmembrane peptide KL₄ accelerates lipid spreading better than its amphipathic isoform KL_{2,3} and the amphipathic peptide WMAP10. KL₄ and WMAP10 are identical to the published covalent structures [21, 22]. KL_{2,3} is a structural isomer of the KL₄ peptide where the Lys residues are separated by two or three Leu residues instead of four as in KL₄, but the number of residues and amino acid compositions are identical. Helical KL_{2,3} exhibits an amphipathic character, in contrast to the mixed nonpolar/polar surface of KL₄ (Fig. 1). Upon reverse-phase HPLC, using short-chain alcohols containing low amounts of water as mobile phases [24], KL₄ elutes about 15 min before KL_{2,3} (data not shown). Already this clear separation suggests that the peptides are helical under the conditions used for HPLC, since they then present surfaces with quite different polarities (Fig. 1). The mixed nonpolar/polar surface of helical KL₄ gives

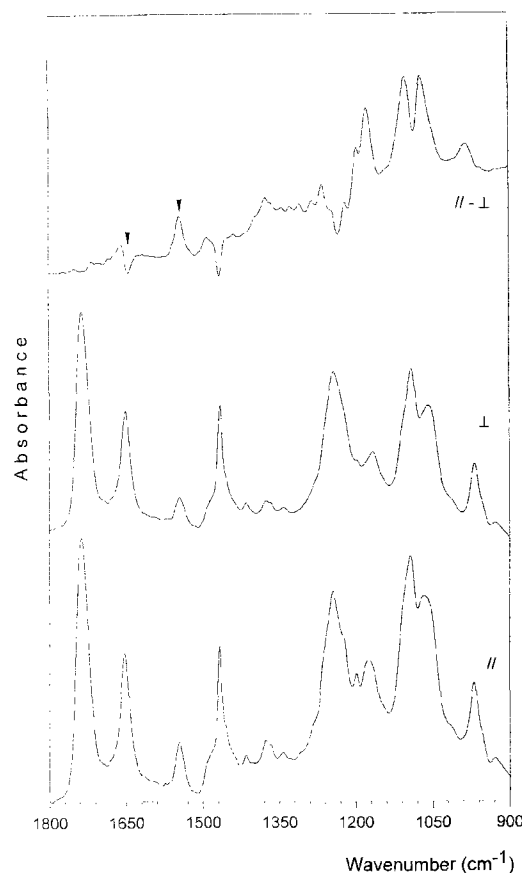


Fig. 3. Dichroic spectrum of deuterated KL_{2,3} in oriented Pam₂GroPCho/PtdGro/PamOH (68:22:9, by mass) bilayers. The dichroic spectrum (top tracing) represents the difference between the spectra recorded with // and ⊥ polarized light. The recorded spectra are drawn with identical ordinate scale; the dichroic spectrum is expanded three-fold in the ordinate direction. The arrows indicate the peptide amide I' band at 1649 cm⁻¹ and the peptide amide II band at 1547 cm⁻¹.

a weaker interaction with the stationary phase compared to the amphipathic surface of KL_{2,3}. Reverse-phase HPLC and CD spectroscopy of other model peptides with different distributions of polar and nonpolar residues gave qualitatively similar results [37].

CD spectroscopy of KL₄ and KL_{2,3} in DodPCho micelles as well as in trifluoroethanol confirms their helical conformations, and a helical content of approximately 85% and 90%, respectively, is estimated ([38] and data not shown). KL₄ behaves as a transmembrane peptide in Pam₂GroPCho/PtdGro, 7:3 (by mass), bilayers, as shown by FTIR spectroscopy [38]. A transmembranous orientation of KL₄ was now observed also in a Pam₂GroPCho/PtdGro/PamOH (68:22:9, by mass) lipid matrix (data not shown). FTIR spectroscopy of a KL_{2,3} film obtained from an ethanol solution shows that nearly all the polypeptide chain adopts a helical conformation with an amide I' (the prime indicates that a H/D exchange has been performed) band centered at 1650 cm⁻¹. In a Pam₂GroPCho/PtdGro/PamOH (68:22:9, by mass) matrix, the orientation of the peptide helix axis with respect to the lipid bilayer can be deduced from the dichroic spectrum (Fig. 3). A dichroic spectrum shows a positive deviation when the dipole moment of the transition is oriented perpendicular to the germanium crystal plane and a negative deviation when the dipole moment is oriented parallel to the germanium crystal plane [30]. The negative deviation observed for

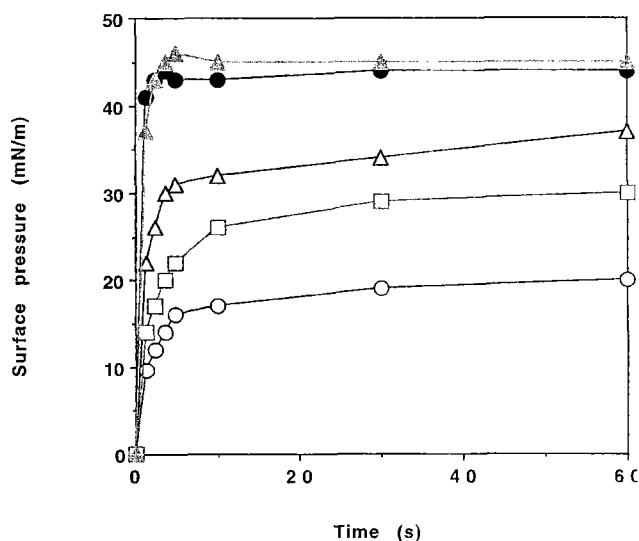


Fig. 4. Surface spreading of transmembrane and amphipathic peptides. Spreading kinetics of 2% (by mass) native SP-C (filled circles), KL₄ (open triangles), KL_{2,3} (open circles), WMAP10 (squares), or SP-C(Leu) (shaded triangles) mixed with Pam₂GroPCho/PtdGro/PamOH, 68:22:9 (by mass), at a phospholipid concentration of 10 mg/ml were analyzed with a Wilhelmy balance. Each data point is the mean of three different recordings.

the KL_{2,3} amide I' band with its maximum located in the helix region, indicates an orientation of the α -helix parallel to the germanium plate, i.e. perpendicular to the lipid acyl chains. As the amide I and amide II polarizations are perpendicular, this orientation is confirmed by the strong positive deviation in the amide II band at 1545 cm⁻¹ corresponding to the α -helix amide II domain [29]. Due to the presence in the α -helix structure of components of different polarization [29], in the present case illustrated by the positive peak at 1659 cm⁻¹, the angle of the helix axis with respect to the lipid bilayer was not determined.

WMA10 (Fig. 1) was designed to be optimally α -helical by facilitating salt bridges between side-chains, neutralising the negative charge of the C-terminus and introducing a negative charge at the N-terminus [23]. By CD spectroscopy we estimate that it contains about 70% helical structure in 50 mM sodium phosphate, pH 6, at room temperature, and about 45% at 90 °C. In 50% aqueous trifluoroethanol 90% helical content is found (data not shown). These values are in agreement with the CD data reported [23]. FTIR spectroscopy of WMA10 gives about 50% helical structure in 5 mM sodium phosphate, pH 7.5. About half of the amide protons remain unexchanged after 17 h of deuteration, which supports the calculated helical content of the peptide. The conformation of the peptide is not modified in the presence of Pam₂GroPCho/PtdGro/PamOH (68:22:9, by mass) lipid matrix. A parallel orientation of the WMA10 helical component with respect to the lipid membrane surface was deduced from polarized FTIR spectra (data not shown).

The spreading kinetics of 2% (by mass) of KL₄, KL_{2,3} or WMA10 mixed with Pam₂GroPCho/PtdGro/PamOH, 68:22:9 (by mass), at an air/water interface were analyzed (Fig. 4). As shown before [22, 38] KL₄ causes rapid spreading with a surface pressure of 37 mN/m after 60 s. In contrast, the amphipathic peptides WMA10 and, in particular, KL_{2,3} are less efficient in improving the spreading kinetics of the lipid mixture (Fig. 4). Mixed with Pam₂GroPCho/PtdGro, 7:3 (by mass), 2% (by mass) of KL₄, KL_{2,3} and WMA10 all showed slower spreading than with PamOH present, but the relative effects of the peptides

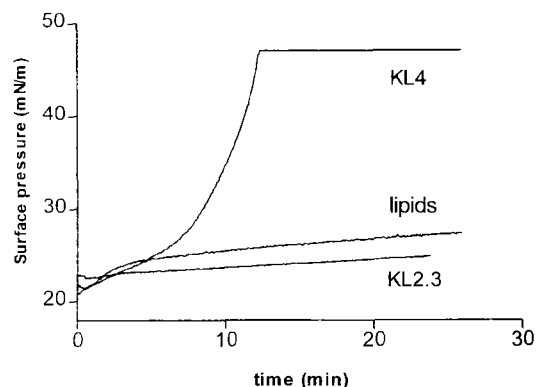


Fig. 5. Monolayer studies of KL₄ and KL_{2,3}. 10% (by mass) of the peptides mixed with Pam₂GroPCho/PamOleGroPGro, 7:3 (by mass), were dissolved in chloroform/methanol, 1:1 (by vol.), and spread on the subphase to a surface pressure of 20.0 mN/m. SUV (40 μ M) made of Pam₂GroPCho/PamOleGroPGro, 9:1 (by mass), were injected through an injection hole into the subphase, and lipid insertion from SUV was initiated at time zero by injection of CaCl₂ solution (to a final concentration of 1.5 mM).

were the same. We were furthermore unable to detect any effects of WMA10 on the spreading kinetics of Pam₂GroPCho (data not shown), which is in contrast to previous results [21].

The effects of KL₄ and KL_{2,3} on insertion of subphase lipids into a preformed lipid/peptide monolayer were determined. The presence of 10% (by mass) of KL₄ in the interfacial Pam₂GroPCho/PamOleGroPGro, 7:3 (by mass), mixture induced lipid insertion and an equilibrium surface pressure of 47 mN/m was achieved after about 12 min (Fig. 5). In contrast, the presence of 10% (by mass) KL_{2,3} in the monolayer did not have any measurable effects on lipid insertion (Fig. 5). Increasing the amount of KL_{2,3} to 30% (by mass) gave nearly identical results as with 10% KL_{2,3} (data not shown).

During preparation of lipid/WMA10 mixtures for FTIR studies, we observed that at a peptide/lipid mass ratio of about 1:10, the mixtures became limpid, indicating that the formation of vesicle structures was impaired. This was not observed with KL_{2,3}-containing or KL₄-containing lipid mixtures. The absorbance at 540 nm of WMA10 mixed with Myr₂GroPCho vesicles at a peptide/lipid mass ratio of 1:10 starts to decrease immediately after addition of peptide and stabilises after about 10 min. It has previously been shown that WMA10 rapidly decreases the turbidity of liposomes [39]. The resultant mixture contains disk structures with an apparent thickness of approximately 30 Å, as determined by electron microscopy [40]. This shows that WMA10 induces formation of Myr₂GroPCho bilayer disks. Similar phenomena have been found for amphipathic helical peptides derived from apolipoproteins [41, 42]. It is noteworthy that WMA10 was originally designed based on apolipoprotein amphipathic helical structures [23].

Poly-Val to poly-Leu substitution in SP-C. Given the result that a transmembrane helix (KL₄) better accelerates lipid spreading than its non-transmembrane isoform (KL_{2,3}; Fig. 4), we aimed to design an analogue of SP-C that mimics its spreading properties but does not exhibit the problems associated with folding of synthetic poly-valyl SP-C analogues [17]. SP-C(Leu) is based on the amino acid sequence of human SP-C, but all Val residues in the α -helical part covering positions 9–34 [10] are replaced with Leu and the palmitoylCys residues at positions 5 and 6 are replaced with Ser (Fig. 1). This peptide folds into a helical conformation without appreciable losses of peptides due

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Table 1. $\gamma_{\min}$ and $\gamma_{\max}$ values of different peptide/lipid mixtures at 10 mg/ml determined in a pulsating bubble surfactometer during cyclic film compression at a rate of 40/min. The values are the mean of four [2% (by mass) SP-C(Leu)] or five [5% SP-C(Leu) and 2% native SP-C] measurements. The values for Curosurf are included for comparison and are taken from [36].

Sample	5th cycle		5 min	
	$\gamma_{\min}$	$\gamma_{\max}$	$\gamma_{\min}$	$\gamma_{\max}$
Pam ₃ GroPCho/PtdGro/PamOH, 68:22:9 (by mass)				
+ 2% SP-C(Leu)	1	43	0	45
+ 5% SP-C(Leu)	0	35	0	43
+ 2% native SP-C	0	42	1	46
Curosurf	0	30	0	30

to aggregation and, notably, also after treatment with neat acids it refolds into a helical conformation which, by CD spectroscopy, is indistinguishable from the non-acid-treated peptide (data not shown). This is in sharp contrast to native SP-C, which upon acid treatment is unfolded into a non-helical structure which does not refold into a helix, but instead forms virtually insoluble aggregates [17]. FTIR spectroscopy of SP-C(Leu) in Pam₃GroPCho/PtdGro/PamOH (68:22:9, by mass) shows that the peptide contains about 84% of α -helical structure. From the dichroic spectrum where a positive deviation in the amide I' band (centered at 1659 cm⁻¹) is observed, we conclude that the helical stretch is oriented parallel to the lipid acyl chains. From the dichroic ratios, we have calculated angles of 22° and 23° with respect to a normal to the crystal plane for the lipid acyl chain axis and the peptide helix axis, respectively. These data confirm an orientation of the helical structure parallel to the lipid acyl chains. Thus SP-C(Leu) exhibits nearly identical overall secondary structure and orientation in a phospholipid bilayer as native SP-C, but it lacks the problems associated with folding of synthetic peptides with the poly-valyl amino acid sequence of native SP-C.

2 g/100 g of SP-C(Leu) mixed with Pam₃GroPCho/PtdGro/PamOH, 68:22:9 (by mass), exhibits excellent spreading properties at an air/water interface, and achieves a surface pressure of 45 mN/m in less than 4 s (Fig. 4). In the pulsating bubble surfactometer the same mixture exhibits a $\gamma_{\min}$ that is close to 0 mN/m, and $\gamma_{\max}$ reaches 43 mN/m (Table 1). SP-C(Leu) behaves practically identical to native SP-C in both these experimental systems (Table 1, Fig. 4). While the $\gamma_{\min}$ values are optimal, the $\gamma_{\max}$ values are higher than those of a surfactant preparation which contains SP-B, SP-C and phospholipids and is in use for treatment of RDS in premature infants (Table 1).

The physiological effects of 2% (by mass) SP-C(Leu) in Pam₃GroPCho/PtdGro/PamOH, 68:22:9 (by mass), were evaluated in a total of 24 preterm rabbit fetuses. 10 of the 13 animals receiving 2.5 ml/kg of the 20 mg/ml suspension of the peptide/lipid mixture, and 8 of 11 of the littermate controls survived the scheduled period of ventilation with normal ECG. pCO₂ in heart blood sampled from surviving animals was 7.7 ± 2.3 kPa in the surfactant-treated group, and 8.3 ± 2.7 kPa in controls. Animals treated with the SP-C(Leu)-based artificial surfactant showed significantly improved lung-thorax compliance. After 60 min of ventilation, the treated animals had compliance values of 0.46 ± 0.05 ml · cm⁻¹ H₂O · kg⁻¹ versus 0.35 ± 0.04 ml · cm⁻¹ H₂O · kg⁻¹ for the non-treated controls, $P < 0.001$.

During preparation of samples for animal experiments we observed that at higher concentrations than about 20 mg/ml the

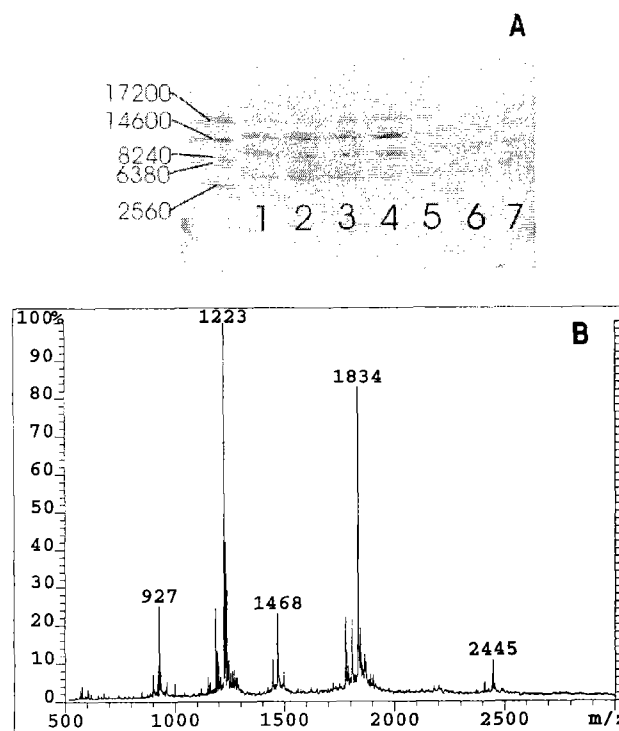


Fig. 6. Oligomerization of SP-C(Leu). (A) SDS/PAGE in a 20% homogeneous gel of SP-C(Leu) dissolved in 2.5% (by mass) SDS without further treatment (lane 1), after treatment at 110°C for 30 min (lane 2), after sonication for 1 min (lane 3), after solubilization of the peptide in 2-propanol (lane 4), 2-propanol/pentane, 1:1 (by vol.), (lane 5), dimethylformamide (lane 6), or dimethylformamide/pentane, 1:1 (by vol.), (lane 7) prior to addition of SDS. The lane furthest to the left shows molecular mass markers with masses of 2560, 6380, 8240, 14600 and 17200 Da. The peptide concentration was about 500 ng/μl. (B) ESI mass spectrum of SP-C(Leu) at a concentration of about 60 ng/μl.

SP-C(Leu)/Pam₃GroPCho/PtdGro/PamOH mixtures started to aggregate. This could not be prevented by increasing the peptide concentration or by heating the samples, and was accompanied by a loss of surfactant activity (data not shown). SDS/PAGE of SP-C(Leu) shows that the peptide can form oligomers (Fig. 6A). In contrast, SDS/PAGE of native SP-C under the same conditions gives only one band corresponding to the monomeric species. The molecular masses of the SP-C(Leu) forms, estimated from the SDS/PAGE bands, are 3.6, 8.3, 14.8 and 22.4 kDa, respectively. This suggests that they correspond to monomers, dimers, tetramers and hexamers of the 3.7-kDa peptide. Also octamers can be observed when larger amounts of SP-C(Leu) are analyzed. The oligomers were observed also when the samples were pretreated in different ways that were expected to reduce self-association (Fig. 6A). Thus, under the conditions used for SDS/PAGE, SP-C(Leu) dimerizes and the dimers can oligomerize further.

ESI mass spectrometry of SP-C(Leu) also showed evidence of oligomeric species. This was concluded from the appearance in the mass spectra of peaks that carry a number of charges corresponding to at least dimeric species, and from the isotope resolution of individual peaks. Shown in Fig. 6B is the ESI mass spectrum of SP-C(Leu). Peaks are observed at m/z 1834, 1223 and 927. These may correspond respectively to the 2+, 3+ and 4+ charged states of monomeric SP-C(Leu) (M , 3666.8). Alternatively they may correspond to the 4+, 6+ and 8+ charge states of dimeric SP-C(Leu). The peaks at m/z 2445 and 1468

cannot correspond to any charge states of the monomer and must correspond to the respective 3+ and 5+ charge states of the dimer, or to more highly charged states of oligomers. The presence of these peaks even at high cone to skimmer voltage differentials confirms the aggregation of SP-C(Leu) molecules. Further evidence for aggregation comes from high-resolution mass spectra where individual isotopic peaks were resolved, allowing charge state assignments indicating the presence of oligomers. It is thus evident from both SDS/PAGE and ESI mass spectrometry that SP-C(Leu) oligomerizes. The different SP-C(Leu) oligomer/monomer ratios observed by SDS/PAGE and ESI mass spectrometry are likely related to the about 10-times higher peptide concentrations used for SDS/PAGE (Fig. 6). Since SP-C(Leu) oligomerizes also at the low concentrations used for ESI and concentrated samples of SP-C(Leu)/Pam₃-GroPCho/PtdGro/PamOH could not be prepared, it seems likely that peptide oligomerization occurs also in Pam₃-GroPCho/PtdGro/PamOH mixtures.

DISCUSSION

Lung surfactant adsorbs and spreads rapidly at the air/liquid interfaces of the lung, and stabilizes terminal airspaces by generation of a surface film that resists compression at low lung volumes. This latter mechanism, important for maintenance of adequate functional residual capacity, probably implies a phase transition of the surfactant film, from a liquid state at equilibrium surface tension to solidification due to selective squeeze-out of unsaturated phospholipid components during surface compression. Pure Pam₃-GroPCho reduces the contractile force of the film to near zero during *in vitro* surface compression but the spreading is much too slow to be effective *in vivo*. Thus, other components have been suggested as spreading agents, especially specific lipids, hydrophobic proteins and calcium ions. Surfactant preparations from natural sources, containing phospholipids and the hydrophobic proteins SP-B and SP-C, have a very fast spreading and reach an equilibrium surface pressure of about 45 mN/m within a few seconds [36]. Rapid lipid spreading is a prerequisite for formulation of an effective surfactant substitute. Pure synthetic lipid mixtures had very low biophysical activities, but addition of 5% native porcine SP-C to the lipid mixtures increased the spreading dramatically and all preparations reached a surface pressure higher than 40 mN/m within 10 min (Fig. 2). However, only the mixture containing PamOH had a spreading rate similar to that of natural surfactant. The basis for the influence of PamOH on the spreading properties is unknown and the natural abundance of palmitic acid in pulmonary surfactant is estimated to be maximally a few grams/100 g, see [6]. SP-B has been shown to specifically interact with PamOH in model systems [20, 43] but also systems containing SP-C as the only protein are significantly influenced by addition of palmitic acid (Fig. 2).

SP-B is built up of several amphipathic helices, while SP-C contains one transmembrane helix. Probably these structural differences also represent different functions of SP-B and SP-C. When present in an interfacial monolayer, SP-B more efficiently than SP-C promotes lipid insertion from subphase vesicles [33], while SP-C apparently better accelerates the spreading of lipids at an air/water interface [7, 17]. Amphipathic KL_{2,3} was designed in order to facilitate a clear-cut comparison with transmembrane KL₄. It is obvious that KL₄ exhibits surface spreading properties superior to those of KL_{2,3} (Figs 4 and 5). Also an SP-C/bacteriorhodopsin hybriide (SP-C/BR) [17] and SP-C(Leu) (Fig. 4), which both are helical transmembrane peptides but with different amino acid sequences, induce rapid spreading kinetics.

Thus, a membrane-spanning α -helix, largely independent of amino acid sequence, seems to promote rapid spreading of lipid mixtures and is therefore one attractive component of artificial surfactants. KL_{2,3} is an amphipathic helix and thus resembles the helices of SP-B, but unlike SP-B it does not promote lipid insertion into an air/water monolayer (Fig. 5). This suggests that a single amphipathic helix cannot replace SP-B, which is a much more complex molecule with several amphipathic helices in a 17.4-kDa homodimeric structure.

Native SP-C contains a poly-valyl region which forms a very stable and regular α -helix [10, 44]. SP-C(Leu), with all helical valyl residues of native SP-C replaced with Leu and the palmitoylated cysteinyls at position 5 and 6 replaced with Ser, exhibits optimal spreading properties (Fig. 4), and folds efficiently into a helical conformation. The better spreading properties of SP-C(Leu) compared with SP-C/BR [17] may be related to its more unpolar surface, which is more similar to that of native SP-C. Recently it has been reported that by fusing poly-Leu segments to the N-terminal six residues of SP-C, analogues with biophysical properties and effects on static lung compliance which are similar to those of native SP-C can be obtained [45].

At a cycling frequency of 40/min natural surfactant preparations have γ_{max} values of about 30 mN/m in the pulsating bubble surfactometer [36], while values around 43 mN/m were obtained for mixtures with SP-C(Leu) (Table 1). The SP-C(Leu)/lipid mixture improved lung function in preterm rabbit neonates, but in contrast to modified natural surfactant [35] it did not convert dynamic compliance to mature levels. This may be due to several factors. Judged from compliance values in controls, the animals used in the present study were less mature than those studied by Sun et al. [35]. Also the concentration and hence the dose of surfactant used now was only 25% of that used for modified natural surfactant in animal experiments [35] or in clinical practice [46] (higher concentrations could not be evaluated because of aggregation). Furthermore, it remains possible that the composition of the SP-C(Leu)-based artificial surfactant is suboptimal, and that SP-B is needed for an optimal surfactant. It is conceivable that SP-B, which is dimeric and may cross-link two lipid layers, can bind subphase lipid vesicles close to the interfacial monolayer and thus facilitate rapid respreading of lipids to the interface upon surface area expansion. Such actions would be expected to reduce the γ_{max} values. Finally, the palmitoyl groups of native SP-C have recently been shown to be important for full surface activity [47, 48] and the lack of palmitoyl groups in SP-C(Leu) could contribute to its suboptimal properties.

The secondary structure and orientation of SP-C(Leu) in a lipid bilayer are similar to those of native SP-C. However, SP-C(Leu), in contrast to native SP-C, undergoes oligomerization both in solution and in the presence of SDS and down to at least low micromolar concentrations (Fig. 6). This is likely not associated with loss of the helical structure since SP-C(Leu) is helical also in SDS micelles (data not shown). The isopropyl groups of the helical valyl residues of native SP-C are closely packed [10] and exhibit restricted rotamer sampling upon molecular dynamics simulations, while Leu22 of native SP-C lacks rotamer restriction [44]. The expected greater flexibility of the isopropyl groups of the leucyl side chains of SP-C(Leu), and their location further away from the helical backbone compared with the valyl isopropyl groups of native SP-C, may facilitate helix-helix interactions that enable higher-order peptide oligomerization. We speculate that oligomerization of SP-C(Leu) is, at least partly, responsible for the difficulties encountered in making SP-C(Leu)/lipid mixtures of higher concentration than about 20 mg/ml, and hence the inferior effects, compared with modified natural surfactant, of such mixtures in preterm rabbit

fetuses. Also recombinant and synthetic SP-C, with poly-valyl sequences, can form dimers and higher molecular mass forms [49]. The mechanisms underlying that phenomenon are, however, probably different from those involved in SP-C(Leu) oligomerization, since apparently also trimers of SP-C were found and removal of the two Cys residues significantly decreased oligomerization [49]. Takei et al. [45] did not report whether oligomerization of the poly-leucyl peptides occurred under their experimental conditions. Attempts are presently being made to avoid the oligomerization of SP-C(Leu) and related analogues by changing the composition of their hydrophobic helical part.

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