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

PROCESSING, STABILITY AND INTERACTIONS OF LUNG SURFACTANT PROTEIN C

Jing Li

Stockholm 2005

Published and printed by Karolinska University Press
Box 200, SE-171 77 Stockholm, Sweden

ISBN 91-7140-582-8

To my family

ABSTRACT

Mature SP-C peptide is a 4.2 kDa transmembrane protein which is uniquely expressed in the alveolar type II cell. Human SP-C is generated via multistep proteolytic cleavage of both the C-terminal and N-terminal regions of proSP-C. The function of SP-C *in vivo* remains unclear, but effects of SP-C on the adsorption, spreading, and stability of lipid films at an air/water interface have been documented in a number of *in vitro* studies.

Infants with inherited deficiency of SP-B and SP-B knock-out mice show neonatal respiratory distress syndrome. SP-B deficiency is associated with accumulation of a processing intermediate, SP-C_i, which was characterized in **paper I**. The molecular weight of SP-C_i, determined by mass spectrometry, is 5458 Da. Determination of the SP-C_i covalent structure revealed a 12-residue N-terminal peptide segment, followed by a 35-residue segment that is identical to mature SP-C. Unlike SP-C, SP-C_i exhibits a very poor ability to promote phospholipid adsorption, gives high surface tension during cyclic film compression, and does not bind lipopolysaccharide (LPS) *in vitro*.

SP-C is composed of a flexible N-terminal end and an α-helical C-terminal part. In **paper II**, the secondary structure and the stability of SP-C_i was investigated. The circular dichroism (CD) spectrum of SP-C_i shows that the dominant structure is α-helical. Unlike SP-C, SP-C_i helix does not unfold or aggregate during several weeks of incubation in aqueous organic solvents. Hydrogen/deuterium exchange experiments showed that 15 amide hydrogen atoms in SP-C_i are protected from exchange for at least several weeks. This number is in agreement with the number of residues spanning the poly-valine part. This leads to the conclusion that the polyvaline part of SP-C_i is locked in a helical conformation for weeks. However, if SP-C_i is incubated in an acidic environment, SP-C unfolds and aggregates into amyloid fibrils like SP-C.

The reasons for the reduced function and more stable α–helix of SP-C_i were studied in **paper III**. The NMR structure of an analogue of SP-C_i, SP-C_i(1-31), shows that the α-helix is extended N-terminally compared to mature SP-C. CD spectroscopy of SP-C_i(1-31) in lipids shows a mixture of helical and extended conformation at pH 6, and a shift to more unordered structure at pH 5. Addition *in trans* of a synthetic dodecapeptide corresponding to the propeptide part of SP-C_i to SP-C results in slower aggregation kinetics, altered amyloid fibril formation, and reduced surface activity of phospholipid-bound SP-C. These data suggest that the propeptide part of SP-C_i prevents helix unfolding by locking the conformation of the N-terminal part of the helix, and that acidic pH results in structural disordering of the region that is proteolytically cleaved to generate SP-C.

SP-C can recognize LPS. In **paper IV**, using synthetic SP-C analogues, it was shown that the capacity of SP-C to bind LPS requires both the hydrophilic N-terminal region and the C-terminal hydrophobic region of the peptide. Using chemically modified LPS and synthetic lipid A analogs, we established that the phosphate residue, when present in α-configuration, is required for the interaction. Finally, more efficient binding between SP-C and LPS was observed in a neutral lipid environment.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numbers:

I. **Jing Li**, Machiko Ikegami, Cheng-Lun Na, Aaron Hamvas, Quentin Espinassous, Richard Chaby, Lawrence M. Nogee, Timothy E. Weaver, and Jan Johansson
N-terminally extended surfactant protein (SP) C isolated from SP-B-deficient children has reduced surface activity and inhibited lipopolysaccharide binding
Biochemistry (2004) **43***,* 3891 – 3898

II. **Jing Li,** Waltteri Hosia, Aaron Hamvas, Johan Thyberg, Hans Jörnvall, Timothy E. Weaver and Jan Johansson
The N-terminal propeptide of lung surfactant protein C is necessary for biosynthesis and prevents unfolding of a metastable α-helix
Journal of Molecular Biology (2004) **338**, 857-862

III. **Jing Li,** Edvards Liepinsh, Andreas Almlén, Johan Thyberg, Tore Curstedt, Hans Jörnvall, and Jan Johansson
Structure and influence on stability and activity of the N-terminal propeptide part of lung surfactant protein C
Submitted

IV. Luis A. Augusto, **Jing Li,** Monique Synguelakis, Jan Johansson, and Richard Chaby
Structural basis for interactions between lung surfactant protein C and bacterial lipopolysaccharide
Journal of Biological Chemistry (2002) **277,** 23484-23492

CONTENTS

ABBREVIATIONS

Three- and one-letter codes for the twenty gene-encoded amino acids.

Amino acid	Three-letter code	One-letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Typ	W
Tyrosine	Tyr	Y
Valine	Val	V

Aβ	Amyloid β-peptide
BAL	Bronchoalveolar lavage
DPC	Dodecylphosphocholine
DPPC	1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine
ER	Endoplasmic reticulum
LPS	Lipopolysaccharide
PAP	Pulmonary alveolar proteinosis
PC	Phosphatidylcholine
PG	Phosphatidylglycerol
POPG	1-palmitoyl-2-oleoyl-*sn*-glycero-phosphoglycerol
PrP	Prion protein
RDS	Respiratory distress syndrome
SP	Surfactant protein

CBS	Captive bubble surfactometer
CD	Circular dichroism
CID	Collision-induced dissociation
EM	Electron microscopy
HPLC	High performance liquid chromatography
H/D	Hydrogen/deuterium
MALDI	Matrix-assisted laser desorption ionization
NMR	Nuclear magnetic resonance
Q-TOF	Quadrupole-time of flight

INTRODUCTION

General introduction

The pulmonary surfactant system has been studied for about 80 years since von Neergaard discovered it in the 1920's. Today, pulmonary surfactant is described as a complex mixture of lipids and proteins which lines the alveolar air/water interface, thereby preventing alveolar collapse at the end of expiration.

The overall lipid composition of surfactant isolated from a number of species is highly conserved (1, 2). The most abundant lipids are phospholipids, which account for approx. 80-90% of total surfactant weight. In addition, cholesterol, triacylglycerols, and free fatty acid are present in surfactant. The most abundant phospholipid species is phosphatidylcholine (PC), which comprises approximately 80%, about half of which is 1,2-dipalmitoyl-*sn*-glycero-3-phoshocholine (DPPC) (3, 4). The acidic phospholipids phosphatidylglycerol (PG) and phosphatidylinostitol (PI) account for 8-15% of the total surfactant phospholipids. The other phospholipids, such as phosphatidylethanolamine (PE), phosphatidylserine (PS) and sphingomyelin, are present in small amounts (1). For some major lipid classes, the role in surfactant has been indicated. DPPC, being saturated and nonfluid at 37 ˚C, is the major component to generate low surface tension. Unsaturated and more fluid phospholipids, for example PG and PI are important for enhancing adsorption of lipids to the air/water interface (5). However, the role of the minor components is less clear and requires further study.

Surfactant proteins make up about 10% (w/w) of total surfactant. So far, four surfactant proteins, surfactant protein (SP)-A, SP-B, SP-C and SP-D have been identified (1, 2). SP-A and SP-D are hydrophilic proteins, which are members of the collectin family and play important roles in host defense mechanisms of the lung (6-8). They interact with various microorganisms and pathogen-derived components in the lung. Both hydrophobic proteins, SP-B and SP-C are believed to be involved in lowering alveolar surface tension by promoting rapid adsorption of lipids to the air/water interface (2, 9).

Respiratory distress syndrome (RDS) is the most frequent respiratory cause of death and morbidity in newborn infants. Deficiency of pulmonary surfactant due to immaturity is the principal cause of the RDS in premature infants (10, 11). Recently, it has been recognized that surfactant deficiency may develop in full-term infants due to

genetic abnormalities in surfactant metabolism (12, 13). RDS can be effectively treated with surfactant preparations which contain phospholipids and hydrophobic surfactant proteins (14). Severe RDS is associated with hereditary SP-B deficiency, which leads to lethal respiratory failure at birth (12, 15-20). The only effective treatment for SP-B deficiency is lung transplantation (16).

SP-C primary and secondary structures

FGIPC$_{pal}$C$_{pal}$PVHLKRLLIVVVVVVLIVVVIVGALLMGL

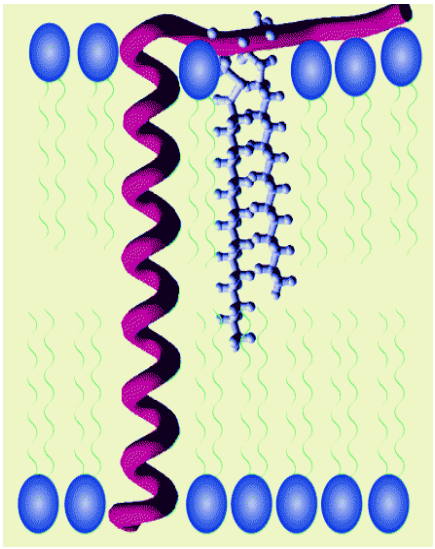

Figure 1. Human surfactant protein C . The peptide is 35 residues long in which residues His9 to Gly34 (underlined) form an ordered α-helix. The length of the helix perfectly matches the phospholipid bilayer. Two palmitoyl groups linked with Cys5 and Cys6 at the N-terminal part of SP-C stablize the helical conformation. Figure adopted from (21)

The primary and secondary structures of SP-C are highly conserved between species (22, 23). Normally, SP-C is composed of 35 amino acids, except dog and bovine SP-C, which are one residue shorter at the N-terminal end. Mature SP-C is one of the most hydrophobic peptides in mammalians. The predominant hydrophobicity of SP-C owes to the high content of Val, Ile and Leu (above 60% of the primary structure). In addition, SP-C is a lipopeptide in which Cys at positions 5 and 6 are acylated with palmitoyl groups (24, 25), except for dog and mink SP-C in which one Cys is replaced by Phe. The presence of acyl chain(s) increases SP-C hydrophobicity. However, the

presence of positively charged residues at the N-terminal end leads to an amphipathic nature of SP-C.

The length (37 Å) of the SP-C α-helix, which covers the residues from 9 to 34, perfectly matches the thickness of DPPC bilayer, strongly suggesting that SP-C is a transmembrane peptide (26). Fourier transform infrared-red spectroscopy of SP-C in DPPC/PG vesicles shows that the insertion of the SP-C in a lipid membrane is associated with an increase of the α-helical content, and the axis of the α-helix is oriented parallel to the lipid acyl chains. In addition, about 70% of the amide hydrogens are hard to exchange with deuterons, indicating a hydrophobic and rigid nature of the SP-C α-helix (27, 28). NMR spectroscopy in aqueous organic solvents of SP-C revealed that the amide protons of residue 10-32 do not exchange during the lifetime of the helix, further indicating that the helix of SP-C is rigid (29).

Amyloid fibril formation

The SP-C α-helix is composed essentially of a long stretch of valine residues, which are branched at the β-carbon and therefore are over-represented in β-sheet and under-represented in α-helix (30). In line with these properties, SP-C amino acid sequence is predicted to form an α-helix in a lipid membrane and a β-strand in a polar environment (31). The highly ordered SP-C α-helix is stable within a lipid membrane. In aqueous organic solvents, the α-helix is thermodynamically unstable and irreversibly transforms into insoluble β-sheet aggregates and finally forms amyloid fibrils (29, 32-36). Synthetic SP-C analogues containing a poly-valine stretch do not fold into helix conformation, and form insoluble aggregates (37, 38). Poly-leucine segments have high helical propensities; leucine has an α-helix/β-strand propensity ratio of 1.2, while the same ratio for valine is 0.46 (39). Substitution by poly-leucine stretch in synthetic SP-C analogues, such as SP-C33 or SP-CLeu, results in a stable α-helix experimentally and by predictions, which are virtually identical to native SP-C in terms of surface activity both *in vivo* and *in vitro* (31, 40, 41). It has been concluded that if the SP-C helix unfolds, the high β-strand propensity will make it less able to refold to helical conformation than to adopt the pathway of β-strand → oligomer → fibril formation (39). In contrast, SP-C33 and SP-CLeu are stable in α-helical conformation. (31, 39) (Fig. 2).

The presence of palmitoyl group(s) not only increases the hydrophobicity of SP-C, but also stabilises the helix conformation. Partial or complete removal of palmitoyl group(s) accelerates SP-C aggregation and decreases the overall α-helical content by about 20% (3, 32, 36, 42). The half life of α-helical dipalmitoylated SP-C is approximately 70 hours, while it is about 35 hours for mono- and nonpalmitoylated SP-C (42).

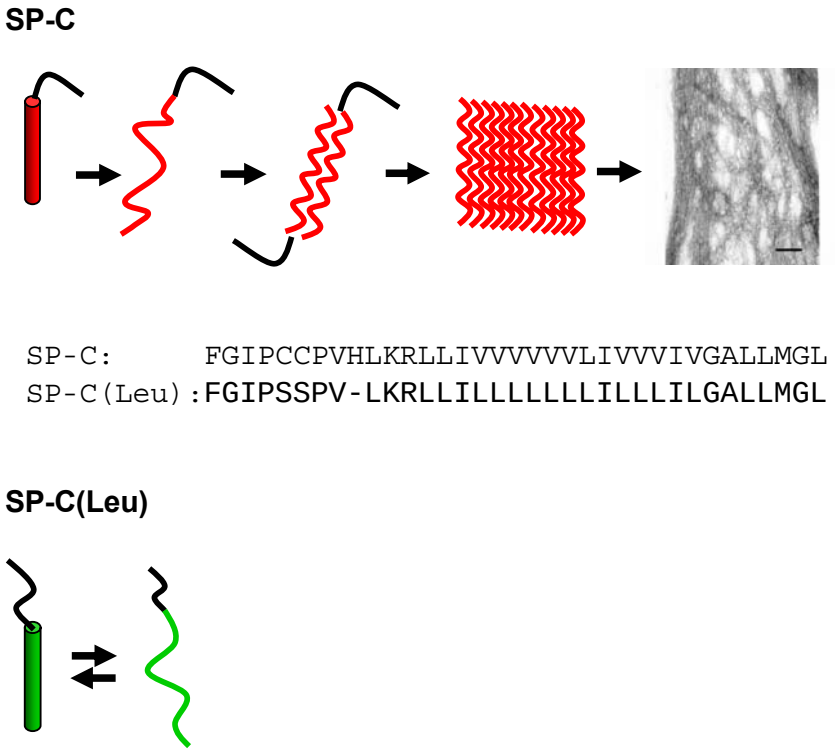

Figure 2. Fibril formation by SP-C. Both SP-C (red) and SP-C(Leu) (green) are in helical conformation in their native states. Monomeric SP-C helix irreversibly converts to a non-helical intermediate which does not refold but aggregates into β-sheet oligomers and forms amyloid fibrils, while SP-C(Leu) refolds into a helix after unfolding. *See (43)*

Aggregation of SP-C is observed under the pathologic condition pulmonary alveolar proteinosis (PAP) (32) (Fig.3). PAP has been recognized for almost half a century since Rosen and co-workers first reported it in 1958 (44) as an excessive accumulation of surfactant lipoprotein in the alveoli, with associated disturbance of pulmonary gas exchange. Nonpalmitoylated or monopalmitoylated SP-C were found in the alveolar space from PAP patients (45), in which the palmitoyl chains could have been cleaved

by reactive oxygen species, such as hydrogen peroxide. The cleavage of palmitoyl group(s), together with disrupted removal of surfactant by alveolar phagocytes, could result in the accumulation of amyloid fibrils in alveolar space in PAP patients (32). However, in healthy individuals SP-C is almost exclusively di-palmitoylated (45).

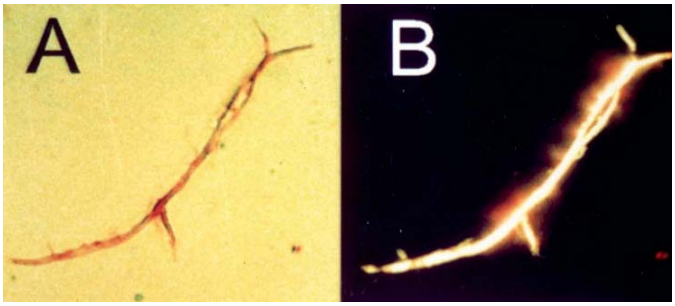

Figure 3. Amyloid fibrils composed of SP-C isolated from patients suffering from pulmonary alveolar proteinosis (PAP). The fibrils were stained with Congo red and observed by bright field microscopy (A) or by polarization microscopy (B). *See (32)*

Amyloid fibrils can be formed from different proteins and are associated with severe diseases. So far, at least 20 diseases are found to be related to amyloid fibrils of proteins. These proteins include the amyloid β-peptide (Aβ) in Alzheimer's disease (46, 47), the prion protein (PrP) in the spongiform encephalophaties (48), and the islet amyloid polypeptide (IAPP) in type 2 diabetes mellitus (49, 50). Although these proteins are completely unrelated in size, amino acid sequence, structure and function, they all have the ability to undergo structural conversions to β-sheet, and subsequently form fibrils. Fibrils are morphologically very similar to each other (47). Amyloid fibrils are unbranched and 5 to 10 nm in diameter (47, 51-54), and can be stained with the dye Congo red and show birefringence under polarized light. X-ray diffraction analysis suggests that the β-sheet runs parallel with the fibril axis, and the constituent β-strands thus run perpendicular to the fibril axis (47). The mechanisms underlying the occurrence of conformational conversion are largely unknown. Interestingly, like SP-C, one helix each of Aβ and PrP are predicted to form β-sheet structure (31).

Biosynthesis, intracellular trafficking and processing of SP-C

SP-C is expressed by only one cell type, the alveolar type II epithelial cell, in lung. Human SP-C is encoded by a single gene that spans approximately 3500 bp on the

short arm of chromosome 8 (55, 56). The gene contains 6 exons and the sixth exon is not translated. The mRNA, about 900 bp, is translated into a 191 or 197 amino acid proprotein (proSP-C) depending on species (23, 56-58). The processing from proSP-C to mature peptide (corresponding to residues 24-58 of proSP-C) includes directional integration into the ER membrane, covalent palmitoylation in the Golgi apparatus, and multi-step proteolytic cleavage of flanking regions from both the N- and C-terminal end (59-62) (Fig. 4).

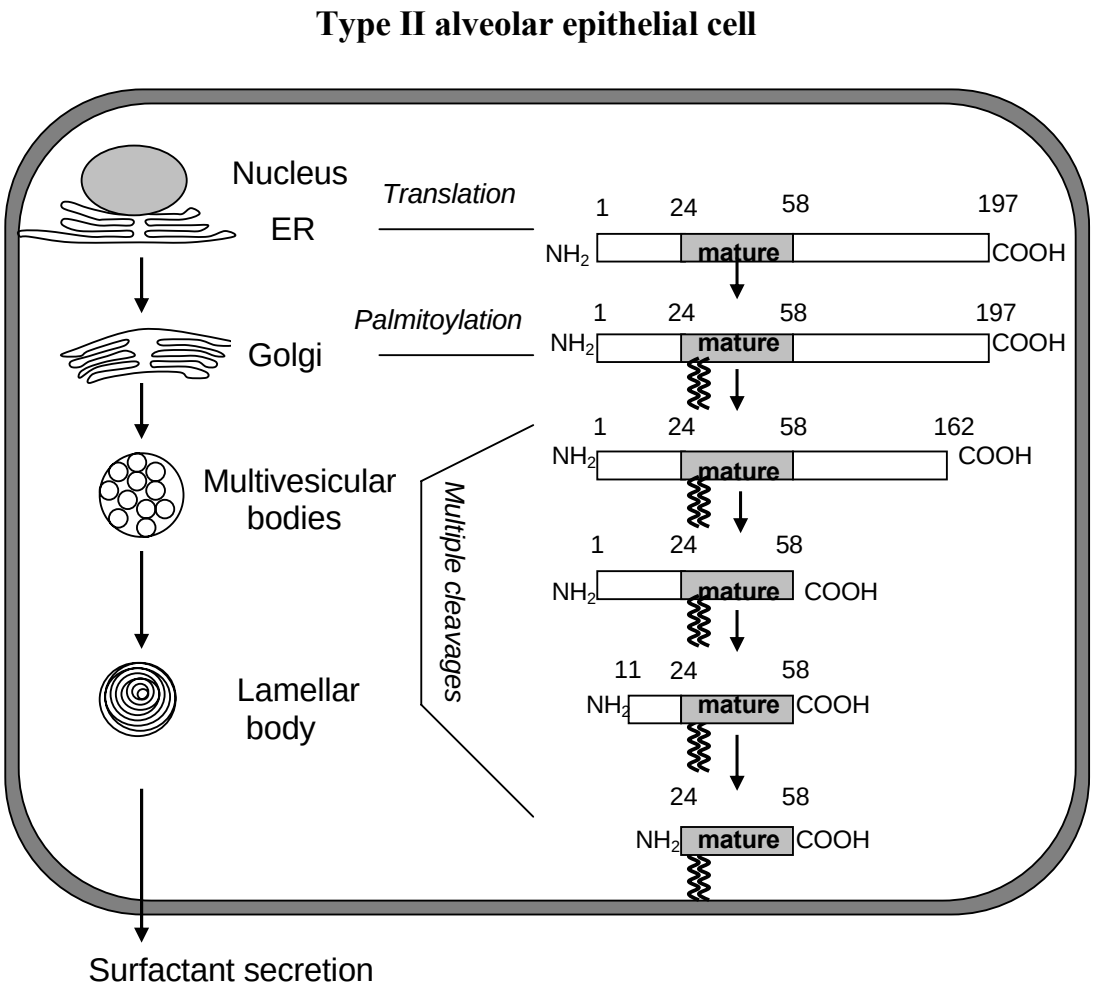

Figure 4. Schematic diagram of SP-C biosynthesis in type II cells. Human proSP-C is synthesized as a 197-amino acid integral transmembrane protein which is processed to the 35-residue mature peptide within the secretory pathway. Proteolytic cleavage starts in the Golgi apparatus and is completed in the lamellar body. Newly synthesized proSP-C in the ER membrane orients the N-terminus to the cytosol, and the C-terminus in the ER lumen. Trafficking of proSP-C from ER to the distal secretory pathway depends on the N-terminal domain of proSP-C.

ProSP-C is an integral transmembrane protein with a molecular weight of 21 kDa. ProSP-C is positioned in ER membrane in type II orientation, meaning that the N-terminal end is located in cytosol and the C-terminal part localizes in the lumen (60, 63-65). These two parts are linked together by the α-helical transmembrane region which corresponds to the mature SP-C part (63, 65). Substitution of two positively charged juxtamembrane residues, Lys34 and Arg35 of proSP-C, by neutral amino acids results in complete reversal of the orientation and total abrogation of post-translational processing, and the protein is retained in the ER (64). Like proSP-C, the partly processed intermediate(s) of proSP-C present in distal intracellular compartments along the biosynthesis pathway are integral membrane proteins.

Palmitoylation of two Cys residues in SP-C occurs in the Golgi apparatus during the biosynthesis pathway, through formation of thioester bonds (66). Palmitoylated proSP-C is transported to the lamellar body, which is a storage form of surfactant lipids and proteins, via multi-vesicular bodies, where multi-step cleavages both at the N- and C-terminal ends occur to generate processing intermediates. Two distinct cleavages apparently take place in the C-terminal region to generate 16 kDa and 7 kDa intermediates, respectively (60, 67). Then, cleavage step(s) in the N-terminal domain form an SP-C intermediate ($SP\text{-}C_i$) with a molecular weight of about 6 kDa (59, 60, 62, 67). The enzymes involved in these proteolytic processes in type II cells are poorly understood, however cathepsin H is known to be involved in the first N-terminal cleavage in the multi-vesicular bodies (68).

The final proteolytic cleavage of proSP-C occurs in the lamellar body which is a lysosome-like secretory granule (59, 67, 69). Fusion of multi-vesicular bodies with lamellar bodies leads to incorporation of SP-C-containing vesicles into densely packed lipid bilayers (69, 70). The contents of lamellar bodies, i.e. all the lipids which forms alveolar surfactant, as well as SP-B and SP-C (1, 69, 71, 72), are excreted by exocytosis into the alveolar space (73-76). There, the lamellar body converts into a lattice of bilayers called tubular myelin (77, 78). Eventually tubular myelin spreads into a monolayer film at the air/water interface, thereby lowering the alveolar surface tension (77, 78). It is estimated that the lamellar body has an acidic internal pH of 5.5 to 6, due to the presence of v-ATPase, which is essential to generate and maintain the acidic environment (79, 80). The acidic subcellular compartments in the distal secretory

pathway appears to be important for the posttranslational proteolysis, since inhibition of proSP-C processing can be observed by inhibitors of organellar acidification (81).

Unlike SP-A and SP-B, the N-terminal region of proSP-C does not contain any classic cleavable signal sequence. Conkright and collaborators showed, with deletion analyses in transiently transfected PC12 cells and mouse type II cells, that the mature SP-C is sufficient for sorting to the regulated secretory pathway, suggesting that mature SP-C contains the targeting signal (74). The correct intracellular trafficking of proSP-C has been reported to be dependent on the N-terminal propeptide (62, 74). Deletion of the region from residues Met10 to Thr18 results in retention of the peptide in ER compartments (62, 74). In contrast, the role of the C-terminal flanking domain remains unclear. Truncation mutants of proSP-C which lacks the entire C-terminal region can be directed to distal compartments in transfected epithelial cells, suggesting that the C-terminal domain is dispensable for intracellular trafficking and secretion (74).

SP-C genetic alterations

SP-C knock-out mice

Targeted disruption of the SP-C gene in one mouse strain (Swiss black outbred mice) results in mild abnormalities of lung function consistent with decreased stability of the surfactant film at low lung volumes (82). However, in another mouse strain (Sv strain mice), SP-C knock-out gives progressive inflammatory lung disease (83). These differences suggest that the phenotype associated with SP-C deficiency may be influenced by genetic or environmental factors. However, unlike SP-B knock-out mice, lack of SP-C is not lethal and SP-C thus does not appear to be critical for surfactant function (Table 1). However, since SP-B knock-out also results in almost complete lack of mature SP-C (see further below), the individual contributions of SP-B and SP-C are difficult to delineate.

SP-C mutations

To date, more than 20 human SP-C gene mutations leading to missense or splice mutations have been detected in patients with interstitial lung disease (84-91). These patients did not have respiratory symptoms at birth, and the age of disease onset is variable (85, 86). All the mutations occur on only one allele. These mutations are believed to affect proSP-C folding and processing (86-88, 90, 92, 93). Most of the mutations are located in the C-terminal flanking region of proSP-C, only one mutation

is localized in the mature peptide region, and no mutations are found in proSP-C N-terminal flank domain (87).

Mutations in the SP-C gene can be classified depending on the involvement of the Brichos domain. The Brichos domain consists of approximately 100 amino acids, and was recently identified in several unrelated proteins linked to different diseases (94). The Brichos-containing proteins include BRI_2 which is linked to familial British and Danish dementia (95, 96); chondromodulin-I linked to chondrosarcoma (97); CA11 linked to stomach cancer (98); and SP-C linked to lung fibrosis. In several of these proteins, the Brichos domain is located in the propeptide region that is removed after proteolytic processing. It has been suggested that the role of this domain could be related to the complex post-translational processing of these proteins (94).

	SP-B deficiency	**SP-C deficiency**
Age of onset	Neonatal	Variable
Typical symptoms	Respiratory failure Airleak and pulmonary hypertension common	Tachypnea Failure to thrive
Inheritance	Recessive	Dominant
Hydrophobic surfactant proteins	SP-B absent Mature SP-C low	Abnormal aggregated proSP-C Mature SP-C low
Pathology	Lethal, RDS	Interstitial lung disease

Table 1 Surfactant protein gene abnormalities and lung disease.

Residues 94-197 in proSP-C, corresponding to exons 4 and 5, contain a Brichos domain. The majority of SP-C mutations linked to lung fibrosis map to the Brichos domain, and these mutations are believed to affect propeptide folding and conformation (99). For example, deletion of exon 4 results in proSP-C misfolding, leading to formation of aggresomes, which in turn induces ER stress and cell apoptosis (84, 85, 87, 93). Clinically, mutations in the Brichos domain result in a

severe pulmonary phenotype (84, 87), while mutations outside this domain lead to a milder clinical course (84, 90, 91). A recombinant C-terminal flanking domain of proSP-C binds to SP-C peptides in β-strand conformation, but not to α-helical SP-C. This rises the interesting possibility that the proSP-C Brichos domain works as a scavenger of unfolded SP-C and thereby prevents β-sheet aggregation (100).

Possible mechanisms underlying that SP-C mutations on one allele cause interstitial lung disease could be that the mutations produce an aberrant proSP-C protein which could interact with non-mutant SP-C, thereby inhibiting production of functionally active SP-C. Alternatively, production of misfolded mutant proSP-C can not be handled by the protein degradation pathway, and it therefore induces ER stress and apoptosis, resulting in interstitial lung disease.

SP-B and SP-B deficiency due to genetic alterations

SP-B

Human SP-B is encoded by a single gene spanning approximately 10 kilobases on the short arm of chromosome 2 (101). SP-B is not only expressed in alveolar type II cells, but also in non-ciliated bronchiolar epithelial cells (Clara cells) (20). However, only in type II cells is proSP-B processed completely to mature SP-B (9, 102) through proteolytic removal of N- and C-terminal flanking domains (19, 73, 76, 102, 103). Mature SP-B is a 79 amino acid polypeptide and contains about 50 % hydrophobic amino acids. Highly conserved Cys residues in proSP-B, present in the N-terminal flanking domain, in the mature SP-B peptide part, and in the C-terminal flanking domain, identify SP-B and the flanking domains as members of the saposin like (SAPLIP) family (104, 105). Other SAPLIP family members include saposins A, B, C, and D, NK-lysin, and a pore forming peptide of *Entamoeba histolytica* (19). The intramolecular disulphide bridges formed by Cys35-46, Cys8-77 and Cys11-71 stabilize the SP-B structure. SP-B normally forms a homodimer through an intermolecular disulfide bond involving Cys48 (106-108) which is not present in other SAPLIPs. SP-B is always associated with surfactant phospholipids. The orientation of SP-B in phospholipids membrane was studied by Vandenbussche and his colleagues. Electrostatic interactions between positively charged residues of SP-B and the anionic headgroups of PG, and the presence of small hydrophobic α-helical peptide stretches slightly inside the bilayers, would maintain SP-B at the membrane surface (109). The

phenotypes of SP-B-deficient human infants and SP-B knockout mice have provided insight into the role of SP-B in the surfactant biosynthetic pathway (see below).

SP-B knock-out mice

The complete lack of SP-B causes lethal respiratory failure at birth (15, 110). Histological analysis of such mice showed a normal lung morphogenesis and no sign of inflammation, but decreased lung volumes and absence of residual volume at the end of expiration was observed. In SP-B knockout mice, no mature lamellar bodies, aberrantly processed SP-C peptides, and enlarged multi-vesicular bodies with small lipid vesicles were observed (110, 111). All these observations suggest that SP-B has a critical role in reduction of alveolar surface tension, maturation of lamellar bodies (possibly via packaging surfactant phospholipids into concentric lamellae), and normal proSP-C processing. Respiratory failure was observed in conditional SP-B knockout mice when the SP-B concentration was reduced to <25% of normal levels (112). Decreased SP-B levels ware associated with low alveolar content of PG, accumulation of misprocessed proSP-C in the air-spaces, increased protein content in bronchoalveolar lavage (BAL) fluid, and altered surfactant activity *in vitro*. Reduction of alveolar SP-B content thus causes surfactant dysfunction and respiratory failure, strongly indicating that SP-B is required for postnatal lung function as well (112).

Hereditary SP-B deficiency in humans

Infants with hereditary SP-B deficiency show neonatal RDS, which is refractory to treatment with exogenous surfactant, and the only effective treatment is lung transplantation (16, 113). In humans, the 121ins2 mutation accounts for about two thirds of the mutant alleles identified to date. This mutation involves the substitution of three bases for one in exon 4 of the gene, corresponding to codon 121 of the SP-B mRNA (114, 115).

Lack of SP-B leads to a number of changes in the metabolism of other surfactant components. The most striking of these is an apparent block in the processing of proSP-C to mature SP-C. In the children with hereditary SP-B deficiency, an incompletely processed SP-C intermediate accumulates (111). In addition, lung tissue and BAL fluid from SP-B-deficient infants contains a reduced amount of PG compared with healthy controls (116).

On ultrastructural examination, the type II cells of SP-B-deficient infants contain few normal-appearing mature lamellar bodies, only inclusions with multiple small vesicles and poorly packed lamellae can be detected (111, 116-118). The phenotype of type II cells in SP-B deficient children is quite similar to that in SP-B knockout mice (15, 110). As a conclusion, SP-B is essential for the formation of lamellar bodies within type II cells and for the normal processing of proSP-C. Partial defects in SP-B can also cause respiratory failure in full-term infants, and decreased amounts of SP-B occur in some term infants with RDS (119-121).

Respiratory distress syndrome

Respiratory disease remains a common cause of neonatal morbidity and mortality, including neonatal RDS, which occurs principally in premature infants (10, 11), as well as in full-term infants due to genetic abnormalities in surfactant metabolism (12, 13, 122). In RDS, the alveoli collapse because of deficiency of pulmonary surfactant. The most effective treatment is replacement with surfactant preparations derived from animal lungs. The components in these preparations are phospholipids and hydrophobic surfactant proteins, SP-B and SP-C (123). Either SP-B or SP-C, when mixed with phospholipids, produces a preparation that readily lowers the surface tension *in vitro* (124).

```
SP-C(Leu): FGIPSSPV-LKRLLILLLLLLLILLLILGALLMGL
SP-C(LKS): FGIPSSPVHLKRLLILKLLLLKILLLKLGALLMGL
SP-C33:      IPSSPVHLKRLKLLLLLLLLILLLILGALLMGL
SP-C/BR:   LRIPCCPVNLKRFYAITTLVAAIAFTLYLSLLLGY
SP-C(1-21):LRIPCCPVNLKRLLVVVVVVV
```

Figure 5. Amino acid sequences of SP-C analogues studied in this thesis. SP-C(Leu) and SP-C33 all contain poly-Val→ poly-Leu substitutions. SP-C(LKS) and SP-C33 contains Lys in the helical part in order to avoid oligomerisation. SP-C/BR is a hybrid between SP-C residues 1-12 and the second transmembrane helix of bacteriorhodoprin. SP-C(1-21) contains the first 21 residues of porcine SP-C.

Surfactant preparations derived from animal lungs are limited in supply, and they may carry infectious agents. Thus, synthetic surfactants are under development. Unlike SP-C, SP-B is too big and structurally complex to be synthesized by organic chemical methods. Synthetic SP-C analogues containing a poly-valine stretch can not fold into a helix conformation (38). Replacement of the poly-valine region with poly-leucine

stretch in synthetic SP-C analogues results in the formation of a stable α-helix (41), and virtually identical surface activity *in vivo* and *in vitro* compared to native SP-C (40) (Fig. 5). SP-C(Leu) oligomerizes into dimers, tetramers, and highly ordered oligomers, which makes SP-C(Leu) difficult to suspend in peptide/lipid mixtures at higher concentrations than 20mg/ml. SP-C(LKS), containing three Lys in the poly-leucine sequence of SP-C(Leu), shows reduced oligomerisation (125), but the activity of SP-C(LKS)/lipids mixtures *in vivo* is insufficient. Finally, SP-C33, with one positive charge in the N-terminal part of the peptide compared to SP-C(Leu), circumvents the oligomerisation problem, and SP-C33/phospholipid mixtures are comparable to modified natural surfactant in an animal model of neonatal RDS (126).

Function of SP-C

SP-C in surfactant function

It is generally believed that SP-B and SP-C are involved in the reduction of the surface tension at the alveolar air/water interface. Numerous *in vitro* studies strongly suggest that SP-B and SP-C are involved in facilitating the rapid adsorption of phospholipids to the interfacial monolayer from the hypophase (127), maintaining a functional interfacial film, stabilizing the surface film at the end of expiration (128, 129), lowering the surface tension by selective DPPC adsorption during film expansion and squeeze-out of fluid components during film compression (thus resisting collapse when compressed) and rapid respreading following film expansion (130-132).

SP-B appears to be the most critical protein for surfactant function, since the effects of SP-C deficiency are less striking than those of SP-B deficiency. The surfactant composition and pool size in SP-C knock-out mice is not significantly different from those of wild type mice. However, SP-B deficiency in both human and animals leads to severe changes in surfactant metabolism and composition (see above). However, surfactant preparations, containing only SP-C and lipids, significantly improve lung function in surfactant deficient animals (41, 133), suggesting an overlap between SP-B and SP-C in the ability to promote and maintain a functional surface film.

SP-C is released from type II cells embedded in a lipid bilayer and the length of the SP-C α-helix is perfectly adopted to span a phospholipids bilayer (26). SP-C can, at least *in vitro*, interact with a phospholipid monolayer and then the axis of the α-helix is tilted to maximize the interaction with the lipids (134). By studying synthetic SP-C analogues,

which contains different amino acid sequences in the helical part, it was concluded that a membrane-spanning α-helix is critical for biophysical activity rather than the primary structure (38, 41, 135).

Lipopolysaccharide (LPS) binding

Infection of the respiratory tract is a frequent cause of lung pathologies, morbidity, and death. Thus in the respiratory tract, recognition of bacterial endotoxin (LPS) is a critical step of the innate host defense system directed against invading pathogens. LPS, located at the outer membrane of gram-negative bacteria, consists of three regions: lipid A, core oligosaccharides, and O side chain. It is generally believed that SP-A and SP-D can interact with LPS: SP-A interacts with the lipid A moiety of LPS (136), and SP-D interacts with the oligosaccharide region of LPS (137). Recently, SP-C was found to exhibit the capacity to bind with LPS (138). The interaction between SP-C and LPS is saturable, temperature-dependent, and related to the concentrations of SP-C and LPS (6, 138). SP-C, when associated with DPPC vesicles, inhibits the binding of LPS to a macrophage cell line (RAW 254.7), the mitogenic effect of LPS on mouse splenocytes, and the LPS-induced production of TNF-α by peritoneal and alveolar macrophages (139). This LPS binding capacity of SP-C may represent another anti-bacterial defense mechanism of the lung. Recently, Charby and colleagues found that CD14 binds to SP-C and the interaction between CD14 and SP-C appears to modify the conformation of CD14, thereby allowing CD14 to bind LPS more efficiently (140).

SCOPE OF THE PRESENT STUDY

- The proSP-C processing intermediate ($SP\text{-}C_i$) that accumulates in SP-B deficiency was isolated and characterized. The identity of this intermediate was previously unknown except that its molecular mass had been estimated to be 6-10 kDa from the electrophoretic mobility. The intermediate was analyzed regarding structural (covalent and secondary structure as well as structural stability) and functional properties (*in vitro* surface properties in combination with phospholipids and LPS binding). This shed light on the possible importance of this intermediate in the pathology associated with SP-B deficiency, and on structural determinants for the stability of the SP-C α-helix.

- Investigation whether the proSP-C N-terminal propeptide, except working as a targeting signal to direct proSP-C to distal compartments in the secretory pathway, can stabilise the SP-C helix. This was aimed to further understand the function of this region in SP-C biosynthesis. Matrix-assisted laser desorption ionization (MALDI) mass spectrometry was applied for analysis of SP-C aggregation, and the effect of the propeptide on SP-C surface activity was investigated by the captive bubble surfactometer (CBS).

- $SP\text{-}C_i$ does not aggregate during at least one month in neutral aqueous organic solvents, while acidic environment destabilizes the $SP\text{-}C_i$ α-helix and makes it aggregate into amyloid-like fibrils within a few days. During its processing, proSP-C is transported from neutral environments (ER and Golgi apparatus) to an acidic environment (lamellar body). We determined an NMR structure of a synthetic $SP\text{-}C_i$ analogue, $SP\text{-}C_i(1\text{-}31)$, and investigated how the secondary structure of $SP\text{-}C_i$ is influenced by pH and different phospholipid environments.

- We aimed to determine what parts of SP-C and LPS are involved in their specific interactions, and to study the role of a lipid environment for SP-C/LPS interactions. For this purpose different analogs of SP-C were synthesized and isolated, and their relative efficiency in binding to LPS was determined.

RESULTS

Paper I. *N-terminally extended surfactant protein (SP) C isolated from SP-B-deficient children has reduced surface activity and inhibited lipopolysaccharide binding*

Infants with inherited deficiency of SP-B and SP-B knock-out mice show neonatal RDS. The proSP-C processing intermediate, SP-C_i, which associates with SP-B deficiency, was characterized in this work. BAL fluid was obtained from SP-$B_{\Delta C}$ mice in which a truncated SP-B proprotein was expressed in the SP-B-/- background. The transgene is not expressed in all type II cells of SP-B-/- mice, resulting in that SP-C_i is generated. Immunogold labeling of large aggregate fraction of alveolar surfactant suggests that SP-C_i associates with surfactant phospholipids and is secreted into the airspaces with the biophysically active form of surfactant. SP-C_i was isolated from patients who were homozygous for the 121ins2 mutation or the c.479G>T mutation. SP-C_i, like SP-C, is highly hydrophobic as judged from its behaviour in reverse-phase HPLC. The molecular weight of SP-C_i, determined by MALDI and quadrupole time-of-flight (Q-TOF) electrospray mass spectrometry, is 5458 Da. Determination of the SP-C_i covalent structure by Edman degradation and by collision-induced dissociation (CID) experiments revealed a 12-residue N-terminal peptide segment, SPPDYSAAPRGR, (positions 12-23 of proSP-C), followed by a 35-residue segment that is identical to mature SP-C (Fig. 6). Two palmitoyl chains are linked to Cys17 and Cys18, Met45 is oxidized, and the C-terminal Leu is methylated. Like for SP-C, oxidation at Met45 and methylation of Leu47 probably occurs during the sample purification (41).

SP-C_i:
SPPDYSAAPRGRFGIPC$_{pal}$C$_{pal}$PVHLKRLLIVVVVVLIVVVIVGALLMGL

Figure 6: Amino acid sequence of SP-C_i. The region in red corresponds to the dodecapeptide elongation, followed by the segment which is identical in sequence to the mature peptide (in black).

Unlike SP-C, SP-C_i exhibits a very poor ability to promote phospholipid adsorption, gives high surface tension during cyclic film compression in captive bubble surfactometer, and does not bind LPS *in vitro*, which are the only two functions described for SP-C to date. During proSP-C processing, SP-C_i N-terminal dodecapeptide segment must be proteolytically removed to generate a biologically

functional peptide. The results of this study indicate that the early postnatal fatal respiratory distress seen in SP-B-deficient children is combined with the near absence of active variants of SP-C.

Paper II. *The N-terminal propeptide of lung surfactant protein C is necessary for biosynthesis and prevents unfolding of a metastable α-helix*

SP-C is composed of a flexible N-terminal end and an α-helical C-terminal part. In this study, the secondary structure and the stability of SP-C_i was investigated. The circular dichroism (CD) spectrum of SP-C_i in 95% ethanol shows that the dominant structure is α-helical, as judged by minimum at 222 nm and 208 nm and a maximum around 195 nm. However, less negative ellipticity at 208 nm was observed for SP-C_i than for SP-C, indicating less α-helix content of SP-C_i. Unlike SP-C, in which the α-helix can irreversibly transform into β-sheet aggregates and form amyloid fibrils, the SP-C_i helix does not unfold or aggregate during several weeks of incubation in aqueous organic solvents, as judged by hydrogen/deuterium (H/D) exchange and MALDI mass spectrometry. H/D exchange experiments showed that about 15 amide hydrogen atoms in SP-C_i are protected from exchange for at least several weeks, while during the same time period, SP-C(Leu) exchanges almost completely. This number of non-exchanged hydrogens is in agreement with the number of residues in the poly-valine part. This leads to the conclusion that the polyvaline part of SP-C_i is protected in a helical conformation for weeks. Moreover, during the first hours of H/D exchange, 10-15 more hydrogens in SP-C_i than in SP-C are partly protected. This number exceeds the number of amide protons in the SP-C_i N-terminal dodecapeptide, suggesting that the presence of the dodecapeptide stabilises the part corresponding to mature SP-C. However, the stability of SP-C_i is pH-dependent. Incubated in an acidic environment, SP-C_i, like SP-C, unfolds and aggregates into amyloid fibrils in one week. Acidic pH may thus weaken the interaction between the dodecapeptide and SP-C.

Paper III. *Structure and influence on stability and activity of the N-terminal propeptide part of lung surfactant protein C*

It is not possible to isolate sufficient amounts of SP-C_i from SP-B-deficient patients to analyse its NMR structure. A peptide corresponding to the N-terminal 31 amino acids of SP-C_i (Fig. 7) was therefore synthesised. In this peptide, two palmitoylated Cys are replaced by Phe, and all Val are replaced by Leu.

SP-C_i(1-31): **SPPDYSAAPRGRFGIPFFPVHLKRLLILLLL**
AlaSP-C_i(1-31): **AAAAAAAAPRGRFGIPFFPVHLKRLLILLLL**

Figure 7: Amino acid sequences of SP-C_i(1-31) and AlaSP-C_i(1-31). In SP-C_i(1-31), two palmitoylated Cys are replaced by Phe since dog and mink SP-C has one palmitoylated Cys replaced by Phe. Substitution from Val to Leu is introduced to prevent β-sheet aggregation.

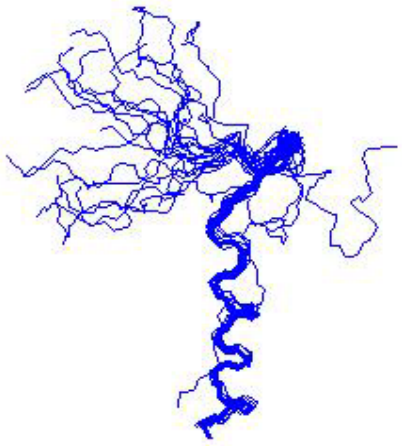

Figure 8: NMR structure of SP-Ci(1-31) in DPC micelles

The NMR structure of SP-C_i(1-31) in dodecylphospocholine (DPC) micelles (Fig. 8) shows a disordered segment from Ser1 to Gly11, a turn from Arg12 to Phe13, an extended stretch involving Gly14 and Ile15, another turn from Pro16 to Phe18, and an α-helix from Pro19 to Leu31. This shows that comparable N-terminal regions of SP-C_i and SP-C have different structures. The helix starts four residue N-terminally in SP-C_i(1-31) compared to a synthetic peptide corresponding to residues 1-17 of mature SP-C in DPC micelles (26). This result strongly indicates that the dodecapeptide extension stabilizes the α-helix. The final cleavage site to generate SP-C from SP-C_i is localized between Arg12 and Phe13, which form a turn structure. CD spectroscopy of SP-C_i(1-31) in microsomal or surfactant lipids shows the presence of both α-helix and β-strand structure at pH 6, and increased random structure was observed as the pH was decreased to 5. Replacement of the N-terminal segment SPPDYS of SP-C_i with AAAAAA (Fig. 7) results in the presence of random and helical structure in microsomal and surfactant lipids at pH 6, and no change was detected when pH was

lowered to 5, indicating that the secondary structure of AlaSP-C_i(1-31) is independent of pH. Incubation of a synthetic dodecapeptide (corresponding to the propeptide part of SP-C_i) with mature SP-C in 95% aqueous ethanol results in slower aggregation kinetics than for SP-C alone. In addition, SP-C incubated with 5-fold molar excess of dodecapeptide did not form amyloid-like fibrils, instead only amorphous aggregate were detected after incubation for 168 hours. These data indicate that the dodecapeptide prevents SP-C_i helix from unfolding. Reduction of the surface activity of phospholipid-bound SP-C by adding 5-fold molar excess of the SP-C_i N-terminal dodecapeptide, strongly indicates the presence of interactions between the dodecapeptide and SP-C as well. Take together, these observations suggest that in the early secretory pathway the N-terminal region of proSP-C not only works as a targeting signal, but also stabilises the α-helical conformation until proSP-C gets to lamellar body, where low pH results in structural disordering of the N-terminal region, thereby allowing proteolytic cleavage to generate SP-C.

Paper IV. *Structural basis for interactions between lung surfactant protein C and bacterial lipopolysaccharide*

SP-C can recognize LPS and the binding between SP-C and LPS is saturable, concentration-, and temperature-dependent (138). In this study, the parts of SP-C and LPS that interact and the role of a lipid environment for SP-C/LPS interactions were investigated. Synthetic SP-C analogues (SP-C(1-21), SP-C(BR), SP-C(LKS), SP-C(Leu) and SP-C(Leu)-di-palm) (Fig. 5) were used for the interaction study. SP-C(1-21) and SP-C/BR have an identical N-terminal hydrophilic region as porcine SP-C, while the other peptides have N-terminal regions identical human SP-C. SP-C(1-21) has a truncated C-terminal region compared to the other peptides, and the C-terminal helical part of SP-C/BR has a completely different amino acid sequence. Palmitoyl groups are present in porcine SP-C, human SP-C, and SP-C(Leu)-di-palm. By comparing the interactions between LPS and SP-C peptides, it was shown that the capacity of SP-C to bind LPS is independent of the presence of palmitoyl groups and requires both the hydrophilic N-terminal region and the C-terminal hydrophobic region. On the other hand, using chemically modified lipopolysaccharides and synthetic lipid A analogs, we established that the phosphate residue of the reducing glucosamine unit, when present in α-configuration, is required for an interaction with SP-C. Additionally, the N-linked fatty acyl chains present on the same glucosamine unit take part in LPS/SP-C interactions. Finally, more efficient binding between SP-C and LPS was

observed in a neutral lipid environment, although binding occurs also in the absence of lipids. This may be explained by that an amphipathic lipid environment can promote an optimal orientation of the two molecules.

DISCUSSION

$SP\text{-}C_i$, an incompletely processed form of proSP-C found in association with SP-B deficiency, is secreted into the alveolar air/water interface with surfactant. The primary structure differences between SP-C and $SP\text{-}C_i$ were investigated in I. A dodecapeptide N-terminal extension distinguishes $SP\text{-}C_i$ from SP-C. In order to analyse the details involved in the stability and functional change induced by the presence of the dodecapeptide (I, II), the NMR structure of $SP\text{-}C_i$(1-31) in DPC micelles was determined (III). A more structured N-terminal region of $SP\text{-}C_i$ was observed, which consists of turn and extended segments from Arg12 to Phe18, followed by an α-helix from Pro19 to Leu31.

The SP-C α-helix is predicted to form β-sheet due to the presence of the poly-valine stretch (31). SP-C is metastable in solution, it converts from helical structure and forms amyloid fibrils within a few days (29, 32, 43, 141). In contrast, an SP-C analogue having a poly-leucine stretch instead of the poly-valine stretch does not aggregate (142), making it likely that the α-helix/β-sheet discordance of SP-C underlies its structural transformation and fibrillation. When $SP\text{-}C_i$ was incubated in aqueous organic solvent for several weeks, no aggregation was observed. In H/D exchange experiment, about 15 hydrogen atoms in $SP\text{-}C_i$ are protected during the same time frame. Taken together with the NMR results of $SP\text{-}C_i$(1-31), these data strongly indicate that N-terminal dodecapeptide stabilises the α-helix in $SP\text{-}C_i$, thereby preventing it from unfolding. Incubation of $SP\text{-}C_i$ in acidified aqueous organic solvents, however, results in the aggregation and formation of fibrils in a few days. This shows that at low pH the stabilising interactions between the propeptide part and SP-C helix are weakened. This is supported by CD experiment showing that lowering pH from 6 to 5 results in a conformational shift to a more disordered structure in $SP\text{-}C_i$(1-31). The shift is likely contributed by the N-terminal segment since the C-terminal unpolar α-helix is buried in the lipid membrane. The correct intracellular trafficking of proSP-C has been reported to depend on the N-terminal propeptide; deletion of the region from residues Met10 to Thr18 of proSP-C results in retention of the peptide in ER compartment (62, 74). $AlaSP\text{-}C_i$(1-31), in which the N-terminal hexapeptide (SPPDYS) is replaced by AAAAAA, shows a combination of random and helix structure independent of pH in CD experiments. This suggests that the helix stabilisation is dependent on the primary structure of N-terminal dodecapeptide of SP-

C_i, This raises the possibility that the propeptide, in addition to acting as a targeting signal, is important for stabilisation of the helix. It is notable that Asp4 in SP-C_i is the only acidic residue in the dodecapeptide, and that two positively charged residues, Lys and Arg, are localized in the N-terminal part of the α-helix. Thus, the helix stabilization by the propeptide could involve electrostatic interactions

The α-helix to β-sheet conversion of SP-C resembles the structural transitions that have been reported to take place in many other proteins and peptides associated with amyloid fibril formation and amyloid diseases, eg, Aβ and PrP are both fibril-forming proteins. Like SP-C, these two peptides contain discordant helical regions which are predicted to form β-strand structure, residues 16-23 in Aβ and helix 2 in PrP (31). Other studies indicate that these regions are important for structural conversion and fibril formation (143, 144). The current understanding of mechanisms involved in fibril formation includes local unfolding of the native protein, intermolecular association leading to increased β-sheet content, and eventually acquisition of cross-β-sheet structure and formation of fibrils (145). Possibly, preferences to adopt β-strand conformation make unfolded discordant helices form β-sheet via protein oligomerisation, and then β-sheet oligomers aggregate into fibrils (43). Stabilisation of fibril-forming segments in native conformation may thus prevent amyloid formation. Stabilisation of transthyretin tetramer by ligands (54) and stabilisation of Aβ α-helix by addition of high amount of trifluoroethanol (146) abrogates fibril formation *in vitro*. Our results showing that the stabilisation of the SP-C helix by the N-terminal dodecapeptide of SP-C_i prevents fibril formation (II, III) give further support to this possibility.

During proSP-C processing in type II cells, it is likely necessary to keep the SP-C α-helix folded in order to prevent aggregation. In the early secretory pathway (ER and Golgi apparatus), the helix appears to be protected by the N-terminal propeptide. In the acidic lamellar body the final proteolytic step to generate SP-C (removal of the dodecapeptide of SP-C_i) takes place. Prevention of SP-C aggregation may thereafter depend on stabilisation by the surrounding phospholipid bilayer. The cleavage of the N-terminal dodecapeptide is dependent on the presence of SP-B, probably because lack of SP-B results in abnormal lamellar bodies (9, 118). For proSP-C, both type II (65) and type III (59) ER membrane orientation have been suggested, but most experimental data support that proSP-C is most likely a type II integral membrane orientation, and

palmitoylation and positively charged residues usually occur on the cytoplasmic side of the membrane (19). With a type II orientation of proSP-C, proteases such as cathepsin H (which was found to co-localize with proSP-C in multivesicular bodies and lamellar bodies, but was not able to produce mature SP-C, suggesting that additional enzymes are involved in the processing of proSP-C (68)) located in the multivesicular bodies can access the C-terminal flanking domain of proSP-C, while the N-terminal flanking domain is not accessible for such proteases since this part is localized on the insides of vesicles. It is believed that SP-B associated with the multivesicular bodies promotes vesicle fusion required to form lamellar bodies (15), which results in the exposure of the contents of internal vesicles to the lumen of the lamellar body (147). The lysis of multivesicular bodies and formation of lamellar bodies, depending on the presence of mature SP-B, thus results in that the N-terminal part of $SP\text{-}C_i$ is exposed to proteases in the lumen of the multivesicular body, which thereby can generate SP-C. Furthermore, complete processing of proSP-C is probably inhibited by loss of an acidic environment in the immature lamellar bodies found in SP-B deficient children or mice (116).

Both SP-B and SP-C influence the formation, refinement, and maintenance of the surfactant film which lines the alveolar interface. Surfactant preparations containing either SP-B or SP-C can restore lung function in surfactant-deficient animals, suggesting an overlap in their surfactant activities (133, 148, 149). Although the phenotype associated with SP-C deficiency is influenced by genetic and/or environmental factors (82, 83), lack of SP-C is not lethal, indicating that SP-B alone can promote surfactant function. $SP\text{-}C_i$ shows low surface activity and LPS binding capacity (I). Lack of significant surface activity for $SP\text{-}C_i$, combined with the almost complete absence of mature SP-C, implies that SP-B deficient children lack active forms of both SP-B and SP-C. This is apparently detrimental for respiratory adaptation at birth, interfering with the lowering of alveolar surface tension and the creation of a stable surfactant film. The SP-C N-terminal region has an intrinsic ability to interact with phospholipid films, mediated by both hydrophobic and electrostatic interactions, even in the absence of the palmitoyl chains attached to this segment in the native protein (150-152). A peptide based on the sequence of the N-terminal segment of SP-C can interact with and insert spontaneously into preformed phospholipid membranes. The ability to interact properly with phospholipids may thus be blocked by the presence of the N-terminal dodecapeptide in $SP\text{-}C_i$. Likewise, reduced SP-C surface activity in CBS experiments by addition of five-fold molar excess of the $SP\text{-}C_i$ N-terminal

dodecapeptide may be caused by disruption of interactions between SP-C N-terminal region and the phospholipids. In IV, it is shown that both the hydrophilic N-terminal and the hydrophobic C-terminal part are required for recognizing LPS. The N-terminal region is likely involved in peptide-LPS interactions whereas the C-terminal region is important for correctly localizing the peptide in a phospholipid bilayer. SP-C_i has virtually no capacity to bind LPS, further supporting the importance of an unblocked N-terminal region of SP-C for peptide/lipid interactions.

CONCLUSIONS

- In both human and mice, SP-B deficiency is associated with accumulation of SP-C_i, a proSP-C processing intermediate, which is secreted into the alveoli as a constituent of surfactant.

- SP-C_i consists of 47 amino acid residues, corresponding to an N-terminal dodecapeptide region (residues 12-23 of proSP-C) followed by the SP-C peptide (residues 24-58 of proSP-C). Like SP-C, two Cys in SP-C_i are palmitoylated. The dominant secondary structure of SP-C_i determined by CD spectroscopy is α-helical.

- The NMR structure of a synthetic peptide based on the sequence of residues 1-31 of SP-C_i shows a structured N-terminal part compared to SP-C, and indicates that the N-terminal region of SP-C_i stabilises the poly-valine helix.

- In a neutral pH environment, the N-terminal dodecapeptide of SP-C_i prevents helix unfolding, while in an acidic environment, stabilising interactions are weakened, resulting in β-sheet aggregation and formation of amyloid-like fibrils.

- The capacity of SP-C to bind LPS requires both the N-terminal and C-terminal region and the presence of lipid membrane makes the interaction more efficient. Regarding LPS, the phosphate residue in α-configuration is required for the interaction with SP-C.

- Both surface activity and LPS binding capacity of SP-C were virtually lost in SP-C_i, indicating that interactions between the N-terminal part of SP-C and phospholipids are interfered by the presence of dodecapeptide extension in SP-C_i.

METHODOLOGY

Some of the methods frequently used in this thesis are briefly presented below. For experimental details, see the original articles.

Mass spectrometry

Mass spectrometry is now one of the most popular and powerful methods for the protein chemist, giving quick, sensitive, and accurate data on the mass of molecules, by ionizing, separating and detecting ions according to their mass-to-charge ratios. Mass spectrometry is also used to determine the structure of molecules.

MALDI mass spectrometry

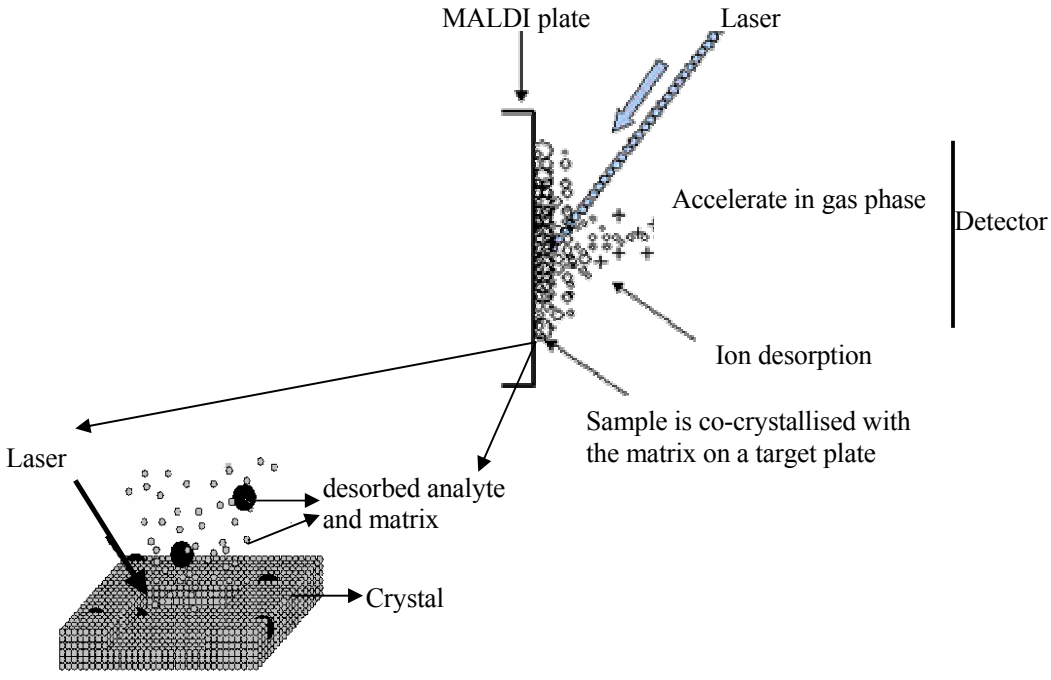

Figure 9. Schematics of the MALDI mass spectrometer

MALDI mass spectrometry was introduced in the late 1980's (153). In this method (Fig. 9) a solid matrix is used, which absorbs light at the wavelength produced by the laser. The matrix, α-cyano-4-hydroxy-cinnamic acid in our studies, works as a proton donor to ionise the sample (154). The sample is mixed with a matrix solution and co-crystallized on a target MALDI plate (155, 156). When the laser beam hits the crystals, the matrix absorbs the laser light energy and desorbs from the plate surface carrying

ionized sample into the gas phase (157, 158). At the time that the laser is pulsed an electric field is applied to the target plate to accelerate the ionised sample towards a time-of-flight mass analyzer. The time for an ion to reach the detector is measured, and used to calculate the mass to charge ratio (*m/z*) (157-159).

Q-TOF mass spectrometry

Electrospray ionization generates ions directly from solution. A sample is sprayed into an electric field from a capillary with an orifice in the micrometer range. Charge accumulates on the sample droplets which are accelerated due to an electrical potential applied between the capillary and a counter electrode. As charged droplets are sprayed into a high vacuum system, the solvent is rapidly evaporated and the droplets brake up into smaller droplets when surface-coulombic forces overcome surface-tension forces (160).

CID can provide information of the amino acid sequence of a peptide (161). After an interesting ion is selected in the first mass analyzer, it is subjected to fragmentation by collision with neutral gas molecules (normally argon). The cleavage commonly occurs in peptide bonds to generate fragmented peptides. A peptide fragment retaining the original C-terminus of the peptide is called a *y-ion*, while those retaining the N-terminus are called *b-ions* (162-164). By comparing the mass difference between two successive *y-ions* or *b-ions*, the primary structure of the peptide can be deduced.

H/D exchange mass spectrometry

H/D exchange mass spectrometry has become a powerful method for high-resolution analyses of protein dynamics since its conception by Kaj Linderstrom-Lang in the 1950s (165).

Exchange of labile hydrogens is a spontaneous chemical process between a protein and the surrounding solvent (166). Proteins contain a variety of exchangeable hydrogens: the hydrogens located on amino acid side chains (-OH, -SH, $-NH_2$, -COOH), and the hydrogens found on backbone amide groups, while hydrogens bound to carbon are unlikely to exchange due to the low polarization of the C-H bond. The intrinsic rate of exchange for a particular hydrogen depends on several factors, including pH,

temperature, solvent accessibility, neighbouring amino acids, and the concentration of the exchange catalyst.

The variation in H/D exchange rate is effected by the diversity of the environment for individual amide hydrogens. Amide hydrogens exposed to deuterated solvent (D_2O) exchange to deuterium rapidly, while the hydrogens buried in the protein interior or involved in hydrogen bond are less likely to exchange. The exchange process is schematically depicted below:

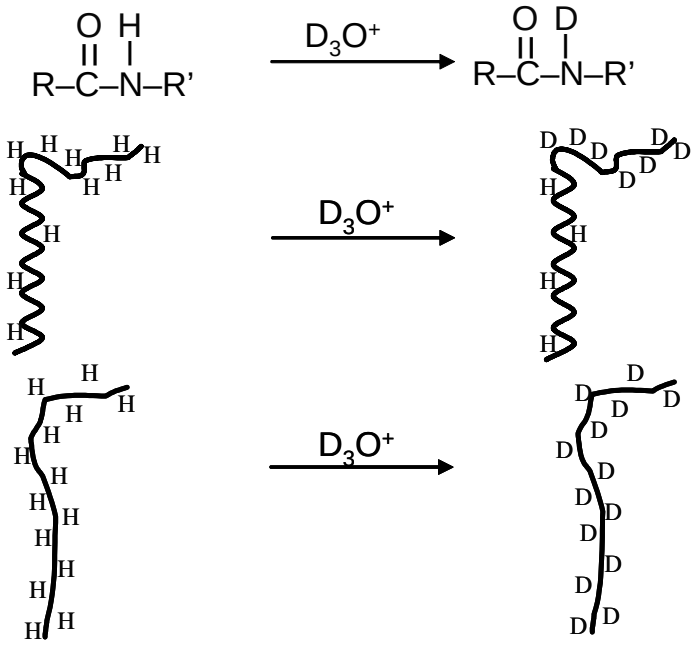

Figure 10. Schematic representation of H/D exchange. Amide hydrogens that are exposed to solvent and not hydrogen-bonded readily undergo chemical exchange with deuterated water. Those amide hydrogens that are shielded from solvent or involved in hydrogen bonds can not exchange with deuterium.

In folded proteins, amide protons in unstructured regions rapidly exchange to deuterium, while those buried in an α-helix or β-sheet can not exchange unless the helix or sheet opens to expose the amide hydrogens to the solvent (167, 168). A shift from lower to higher molecular weight induced by deuterium incorporation can be detected by mass spectrometry. Hence, H/D exchange is an efficient method to assay for the conformational properties of proteins.

Circular dichroism spectroscopy

CD is the difference between the absorption of left- and right- handed circularly polarized light, which interact differently with stereospecific L-amino acids, and is measured as a function of wavelength. CD is measured as a quantity called mean residue ellipticity. It has been extensively applied to the structural characterization of peptides. It has been shown that CD spectra between 260 and approximately 180 nm can be analyzed for the different secondary structural types (169, 170) (Fig. 11). The application of CD for conformational studies in peptides (and proteins) can be largely grouped into monitoring conformational changes and estimation of secondary structural content.

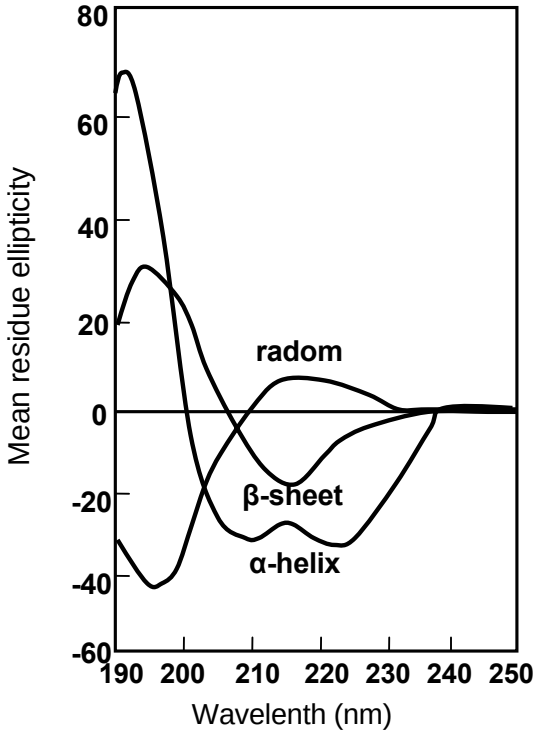

Figure 11. Circular dichroism spectra of α-helix, β-sheet and random conformation. α-helix has double minimum at 208 and 222 and maximum at 192, β-sheet has a minimum at 217 and a maximum at 195 nm, while random structure has a minimum around 197 nm and a maximum at 220 nm.

Captive bubble surfactometer

CBS was introduced by Schürch and co-workers in1989 (171). In this apparatus (Fig. 12), an air bubble is injected into a chamber filled with agarose gel. Subsequently, the sample solution is introduced and adsorbed at the air–agarose interface. Bubble volume is controlled by varying the pressure in the sample chamber. As bubble volume is reduced, the surface area is reduced and the surface tension of the surfactant film at the

bubble surface falls. The bubble shape changes depending on the surface tension, from more spherical to an oval shape. During the process, air bubble surface area and the surface tension calculated from the bubble shape are recorded by a computer (172).

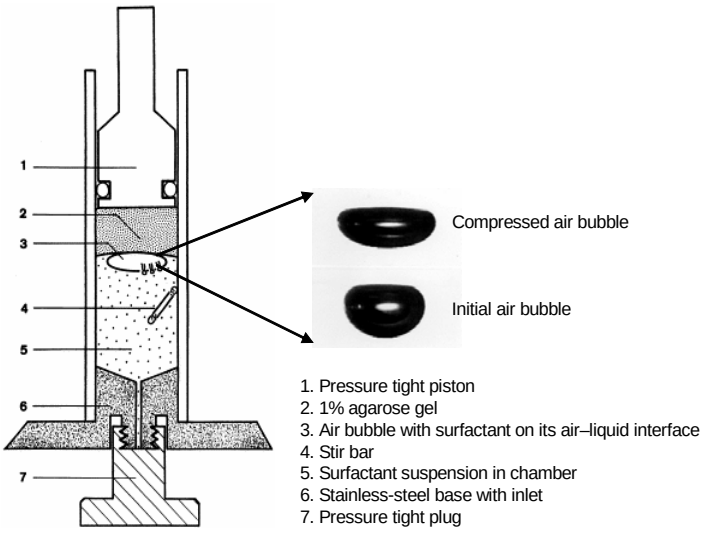

Figure 12. The captive bubble surfactometer. A bubble of atmospheric air 2–7 mm in diameter at initial time, covered by a phospholipid layer, is compressed and expanded stepwise by the changing pressure produced by the relative movement between piston and chamber, *see (172) for details*.

ACKNOWLEDGEMENTS

The presented work in this thesis would never have been possible without help and support from lots of people. In particular I would like to express my sincere gratitude to:

Jan Johansson, my supervisor, who gave me the opportunity to perform my Ph.D study in MBB and introduced me to the field of biochemistry. Without his never-failing support and contagious enthusiasm for hard work, this study would never have been possible. Thank you for careful and critical review of the thesis. You are an excellent tutor.

Professor Hans Jörnvall, head of Chemistry I, for providing excellent working facilities.

Co-authors in the articles, Machiko Ikegami, Cheng-Lun Na, Aaron Hamvas, Quentin Espinassous, Lawrence M. Nogee, Luis A. Augusto, Monique Synguelakis, Tore Curstedt, especially Timothy E. Weaver, Richard Chaby, Johan Thyberg and Edvards Liepinsh for scientific discussion and valuable advise on publications and manuscript.

All the members (past and present) in Janne's group: Waltteri Hosia for showing me how to use mass spectrometry, Andreas Almlen for lots of help on CBS experiment, Kerstin Nordling for your excellent technical assistance, Anna Bergman, Anna Päiviö, Anna Rising, Charlotte Nerelius, Hanna Johansson, Margarea Stark, Stefan Grip and Yuqin Wang for helpful and funny talks and encouragement about everything, anytime, anywhere.

Ingegerd Nylander for invaluable administrative assistance.

Marie Ståhlberg, Carina Palmberg, Ella Cederlund and Irene Byman for all practical help at the lab.

All the members at Chemistry I in Karolinska Institutet and all the members at Department of Molecular Biosciences in Swedish University of Agricultural Sciences.

Professor Tomas Ekström for his kindly recommendation.

I would like to thank all my friends for the warm and friendly atmosphere over the years and all activities we did together.

My parents: Zhiqiang Li and Sumei Meng for all your love and constant encouragement throughout my life.

My parents in law: Qimin Zhang and Yuanli Zhu for their continuous support, care and constructive suggestions through the years.

My lovely sister Tao and her husband Tiejun, for sharing every piece of happiness for the moments we have spent together.

Yin and Tomas, for the unforgettable family time we have had.
Finally, thanks for my husband, Fan Zhang, you are the foundation upon which I build my life.

Thank you all very much.

REFERENCES

1. **Goerke J** 1998 Pulmonary surfactant: functions and molecular composition. Biochim Biophys Acta 1408:79-89
2. **Johansson J, Curstedt T** 1997 Molecular structures and interactions of pulmonary surfactant components. Eur J Biochem 244:675-93
3. **Creuwels LA, Demel RA, van Golde LM, Benson BJ, Haagsman HP** 1993 Effect of acylation on structure and function of surfactant protein C at the air-liquid interface. J Biol Chem 268:26752-8
4. **Kahn MC, Anderson GJ, Anyan WR, Hall SB** 1995 Phosphatidylcholine molecular species of calf lung surfactant. Am J Physiol 269:L567-73
5. **Meban C** 1981 Effect of lipids and other substances on the adsorption of dipalmitoyl phosphatidylcholine. Pediatr Res 15:1029-31
6. **Chaby R, Garcia-Verdugo I, Espinassous Q, Augusto LA** 2005 Interactions between LPS and lung surfactant proteins. J Endotoxin Res 11:181-5
7. **Garcia-Verdugo I, Sanchez-Barbero F, Soldau K, Tobias PS, Casals C** 2005 Interaction of surfactant protein A (SP-A) with bacterial rough lipopolysaccharide (Re-LPS)and effects of SP-A on the binding of Re-LPS to CD14 and LPS binding protein. Biochem J 391:115-24
8. **Senft AP, Korfhagen TR, Whitsett JA, Shapiro SD, LeVine AM** 2005 Surfactant protein-D regulates soluble CD14 through matrix metalloproteinase-12. J Immunol 174:4953-9
9. **Whitsett JA, Weaver TE** 2002 Hydrophobic surfactant proteins in lung function and disease. N Engl J Med 347:2141-8
10. **Zimmermann LJ, Janssen DJ, Tibboel D, Hamvas A, Carnielli VP** 2005 Surfactant metabolism in the neonate. Biol Neonate 87:296-307
11. **Lynch RG** 2004 Surfactant and RDS in premature infants. Faseb J 18:1624
12. **Nogee LM** 1998 Genetics of the hydrophobic surfactant proteins. Biochim Biophys Acta 1408:323-33
13. **Haataja R, Marttila R, Uimari P, Lofgren J, Ramet M, Hallman M** 2001 Respiratory distress syndrome: evaluation of genetic susceptibility and protection by transmission disequilibrium test. Hum Genet 109:351-5
14. **Hallman M, Glumoff V, Ramet M** 2001 Surfactant in respiratory distress syndrome and lung injury. Comp Biochem Physiol A Mol Integr Physiol 129:287-94
15. **Clark JC, Wert SE, Bachurski CJ, et al.** 1995 Targeted disruption of the surfactant protein B gene disrupts surfactant homeostasis, causing respiratory failure in newborn mice. Proc Natl Acad Sci U S A 92:7794-8
16. **Hamvas A, Nogee LM, Mallory GB, Jr., et al.** 1997 Lung transplantation for treatment of infants with surfactant protein B deficiency. J Pediatr 130:231-9
17. **Nogee LM, Garnier G, Dietz HC, et al.** 1994 A mutation in the surfactant protein B gene responsible for fatal neonatal respiratory disease in multiple kindreds. J Clin Invest 93:1860-3
18. **Wilder MA** 2004 Surfactant protein B deficiency in infants with respiratory failure. J Perinat Neonatal Nurs 18:61-7
19. **Weaver TE, Conkright JJ** 2001 Function of surfactant proteins B and C. Annu Rev Physiol 63:555-78
20. **Whitsett JA, Nogee LM, Weaver TE, Horowitz AD** 1995 Human surfactant protein B: structure, function, regulation, and genetic disease. Physiol Rev 75:749-57
21. **Robertson B, Johansson J, Curstedt T** 2000 Synthetic surfactants to treat neonatal lung disease. Mol Med Today 6:119-24
22. **Johansson J, Jornvall H, Eklund A, Christensen N, Robertson B, Curstedt T** 1988 Hydrophobic 3.7 kDa surfactant polypeptide: structural characterization of the human and bovine forms. FEBS Lett 232:61-4
23. **Glasser SW, Korfhagen TR, Bruno MD, Dey C, Whitsett JA** 1990 Structure and expression of the pulmonary surfactant protein SP-C gene in the mouse. J Biol Chem 265:21986-91

24. **Curstedt T, Johansson J, Persson P, et al.** 1990 Hydrophobic surfactant-associated polypeptides: SP-C is a lipopeptide with two palmitoylated cysteine residues, whereas SP-B lacks covalently linked fatty acyl groups. Proc Natl Acad Sci U S A 87:2985-9
25. **Johansson J** 1998 Structure and properties of surfactant protein C. Biochim Biophys Acta 1408:161-72
26. **Johansson J, Szyperski T, Wuthrich K** 1995 Pulmonary surfactant-associated polypeptide SP-C in lipid micelles: CD studies of intact SP-C and NMR secondary structure determination of depalmitoyl-SP-C(1-17). FEBS Lett 362:261-5
27. **Pastrana B, Mautone AJ, Mendelsohn R** 1991 Fourier transform infrared studies of secondary structure and orientation of pulmonary surfactant SP-C and its effect on the dynamic surface properties of phospholipids. Biochemistry 30:10058-64
28. **Vandenbussche G, Clercx A, Curstedt T, Johansson J, Jornvall H, Ruysschaert JM** 1992 Structure and orientation of the surfactant-associated protein C in a lipid bilayer. Eur J Biochem 203:201-9
29. **Szyperski T, Vandenbussche G, Curstedt T, Ruysschaert JM, Wuthrich K, Johansson J** 1998 Pulmonary surfactant-associated polypeptide C in a mixed organic solvent transforms from a monomeric α-helical state into insoluble β-sheet aggregates. Protein Sci 7:2533-40
30. **Johansson J, Szyperski T, Curstedt T, Wuthrich K** 1994 The NMR structure of the pulmonary surfactant-associated polypeptide SP-C in an apolar solvent contains a valyl-rich α-helix. Biochemistr 33:6015-23
31. **Kallberg Y, Gustafsson M, Persson B, Thyberg J, Johansson J** 2001 Prediction of amyloid fibril-forming proteins. J Biol Chem 276:12945-50
32. **Gustafsson M, Thyberg J, Naslund J, Eliasson E, Johansson J** 1999 Amyloid fibril formation by pulmonary surfactant protein C. FEBS Lett 464:138-42
33. **Luy B, Diener A, Hummel RP, Sturm E, Ulrich WR, Griesinger C** 2004 Structure and potential C-terminal dimerization of a recombinant mutant of surfactant-associated protein C in chloroform/methanol. Eur J Biochem 271:2076-85
34. **Wustneck N, Wustneck R, Perez-Gil J, Pison U** 2003 Effects of oligomerization and secondary structure on the surface behavior of pulmonary surfactant proteins SP-B and SP-C. Biophys J 84:1940-9
35. **Kovacs H, Mark AE, Johansson J, van Gunsteren WF** 1995 The effect of environment on the stability of an integral membrane helix: molecular dynamics simulations of surfactant protein C in chloroform, methanol and water. J Mol Biol 247:808-22
36. **Dluhy RA, Shanmukh S, Leapard JB, Kruger P, Baatz JE** 2003 Deacylated pulmonary surfactant protein SP-C transforms from α-helical to amyloid fibril structure via a pH-dependent mechanism: an infrared structural investigation. Biophys J 85:2417-29
37. **Johansson J, Gustafsson M, Palmblad M, Zaltash S, Robertson B, Curstedt T** 1998 Synthetic surfactant protein analogues. Biol Neonate 74 Suppl 1:9-14
38. **Johansson J, Nilsson G, Stromberg R, Robertson B, Jornvall H, Curstedt T** 1995 Secondary structure and biophysical activity of synthetic analogues of the pulmonary surfactant polypeptide SP-C. Biochem J 307:535-41
39. **Hosia W, Johansson J, Griffiths WJ** 2002 Hydrogen/deuterium exchange and aggregation of a polyvaline and a polyleucine α-helix investigated by matrix-assisted laser desorption ionization mass spectrometry. Mol Cell Proteomics 1:592-7
40. **Palmblad M, Johansson J, Robertson B, Curstedt T** 1999 Biophysical activity of an artificial surfactant containing an analogue of surfactant protein (SP)-C and native SP-B. Biochem J 339:381-6
41. **Nilsson G, Gustafsson M, Vandenbussche G, et al.** 1998 Synthetic peptide-containing surfactants--evaluation of transmembrane versus amphipathic

helices and surfactant protein C poly-valyl to poly-leucyl substitution. Eur J Biochem 255:116-24
42. **Gustafsson M, Griffiths WJ, Furusjo E, Johansson J** 2001 The palmitoyl groups of lung surfactant protein C reduce unfolding into a fibrillogenic intermediate. J Mol Biol 310:937-50
43. **Johansson J** 2003 Molecular determinants for amyloid fibril formation: lessons from lung surfactant protein C. Swiss Med Wkly 133:275-82
44. **Rosen SH, Castleman B, Liebow AA** 1958 Pulmonary alveolar proteinosis. N Engl J Med 258:1123-42
45. **Voss T, Schafer KP, Nielsen PF, et al.** 1992 Primary structure differences of human surfactant-associated proteins isolated from normal and proteinosis lung. Biochim Biophys Acta 1138:261-7
46. **Johansson J, Weaver TE, Tjernberg LO** 2004 Proteolytic generation and aggregation of peptides from transmembrane regions: lung surfactant protein C and amyloid beta-peptide. Cell Mol Life Sci 61:326-35
47. **Sunde M, Serpell LC, Bartlam M, Fraser PE, Pepys MB, Blake CC** 1997 Common core structure of amyloid fibrils by synchrotron X-ray diffraction. J Mol Biol 273:729-39
48. **Pan KM, Baldwin M, Nguyen J, et al.** 1993 Conversion of α-helices into β-sheets features in the formation of the scrapie prion proteins. Proc Natl Acad Sci U S A 90:10962-6
49. **Tomita T** 2005 Amyloidosis of pancreatic islets in primary amyloidosis (AL type). Pathol Int 55:223-7
50. **Sumner Makin O, Serpell LC** 2004 Structural characterisation of islet amyloid polypeptide fibrils. J Mol Biol 335:1279-88
51. **Harper JD, Wong SS, Lieber CM, Lansbury PT, Jr.** 1999 Assembly of Aβ amyloid protofibrils: an in vitro model for a possible early event in Alzheimer's disease. Biochemistry 38:8972-80
52. **Goldsbury C, Kistler J, Aebi U, Arvinte T, Cooper GJ** 1999 Watching amyloid fibrils grow by time-lapse atomic force microscopy. J Mol Biol 285:33-9
53. **Kayed R, Bernhagen J, Greenfield N, et al.** 1999 Conformational transitions of islet amyloid polypeptide (IAPP) in amyloid formation in vitro. J Mol Biol 287:781-96
54. **Miroy GJ, Lai Z, Lashuel HA, Peterson SA, Strang C, Kelly JW** 1996 Inhibiting transthyretin amyloid fibril formation via protein stabilization. Proc Natl Acad Sci U S A 93:15051-6
55. **Fisher JH, Emrie PA, Drabkin HA, et al.** 1988 The gene encoding the hydrophobic surfactant protein SP-C is located on 8p and identifies an EcoRI RFLP. Am J Hum Genet 43:436-41
56. **Glasser SW, Korfhagen TR, Perme CM, Pilot-Matias TJ, Kister SE, Whitsett JA** 1988 Two SP-C genes encoding human pulmonary surfactant proteolipid. J Biol Chem 263:10326-31
57. **Warr RG, Hawgood S, Buckley DI, et al.** 1987 Low molecular weight human pulmonary surfactant protein (SP5): isolation, characterization, and cDNA and amino acid sequences. Proc Natl Acad Sci U S A 84:7915-9
58. **Fisher JH, Shannon JM, Hofmann T, Mason RJ** 1989 Nucleotide and deduced amino acid sequence of the hydrophobic surfactant protein SP-C from rat: expression in alveolar type II cells and homology with SP-C from other species. Biochim Biophys Acta 995:225-30
59. **Vorbroker DK, Voorhout WF, Weaver TE, Whitsett JA** 1995 Posttranslational processing of surfactant protein C in rat type II cells. Am J Physiol 269:L727-33
60. **Beers MF, Kim CY, Dodia C, Fisher AB** 1994 Localization, synthesis, and processing of surfactant protein SP-C in rat lung analyzed by epitope-specific antipeptide antibodies. J Biol Chem 269:20318-28
61. **Beers MF, Lomax C** 1995 Synthesis and processing of hydrophobic surfactant protein C by isolated rat type II cells. Am J Physiol 269:L744-53
62. **Johnson AL, Braidotti P, Pietra GG, et al.** 2001 Post-translational processing of surfactant protein-C proprotein: targeting motifs in the NH(2)-terminal

flanking domain are cleaved in late compartments. Am J Respir Cell Mol Biol 24:253-63
63. **Russo SJ, Wang W, Lomax CA, Beers MF** 1999 Structural requirements for intracellular targeting of SP-C proprotein. Am J Physiol 277:L1034-44
64. **Mulugeta S, Beers MF** 2003 Processing of surfactant protein C requires a type II transmembrane topology directed by juxtamembrane positively charged residues. J Biol Chem 278:47979-86
65. **Keller A, Eistetter HR, Voss T, Schafer KP** 1991 The pulmonary surfactant protein C (SP-C) precursor is a type II transmembrane protein. Biochem J 277:493-9
66. **Vorbroker DK, Dey C, Weaver TE, Whitsett JA** 1992 Surfactant protein C precursor is palmitoylated and associates with subcellular membranes. Biochim Biophys Acta 1105:161-9
67. **Solarin KO, Wang WJ, Beers MF** 2001 Synthesis and post-translational processing of surfactant protein C. Pediatr Pathol Mol Med 20:471-500
68. **Brasch F, Ten Brinke A, Johnen G, et al.** 2002 Involvement of cathepsin H in the processing of the hydrophobic surfactant-associated protein C in type II pneumocytes. Am J Respir Cell Mol Biol 26:659-70
69. **Weaver TE, Na CL, Stahlman M** 2002 Biogenesis of lamellar bodies, lysosome-related organelles involved in storage and secretion of pulmonary surfactant. Semin Cell Dev Biol 13:263-70
70. **Dietl P, Haller T** 2005 Exocytosis of lung surfactant: from the secretory vesicle to the air-liquid interface. Annu Rev Physiol 67:595-621
71. **Oosterlaken-Dijksterhuis MA, van Eijk M, van Buel BL, van Golde LM, Haagsman HP** 1991 Surfactant protein composition of lamellar bodies isolated from rat lung. Biochem J 274:115-9
72. **Chander A** 1989 Regulation of lung surfactant secretion by intracellular pH. Am J Physiol 257:L354-60
73. **Voorhout WF, Weaver TE, Haagsman HP, Geuze HJ, Van Golde LM** 1993 Biosynthetic routing of pulmonary surfactant proteins in alveolar type II cells. Microsc Res Tech 26:366-73
74. **Conkright JJ, Bridges JP, Na CL, et al.** 2001 Secretion of surfactant protein C, an integral membrane protein, requires the N-terminal propeptide. J Biol Chem 276:14658-64
75. **Haller T, Ortmayr J, Friedrich F, Volkl H, Dietl P** 1998 Dynamics of surfactant release in alveolar type II cells. Proc Natl Acad Sci U S A 95:1579-84
76. **Weaver TE, Whitsett JA** 1989 Processing of hydrophobic pulmonary surfactant protein B in rat type II cells. Am J Physiol 257:L100-8
77. **Nakamura H, Tonosaki A, Washioka H, Takahashi K, Yasui S** 1985 Monomolecular surface film and tubular myelin figures of the pulmonary surfactant in hamster lung. Cell Tissue Res 241:523-8
78. **Perez-Gil J, Keough KM** 1998 Interfacial properties of surfactant proteins. Biochim Biophys Acta 1408:203-17
79. **Chander A, Johnson RG, Reicherter J, Fisher AB** 1986 Lung lamellar bodies maintain an acidic internal pH. J Biol Chem 261:6126-31
80. **Chander A** 1992 Dicyclohexylcarbodiimide and vanadate sensitive ATPase of lung lamellar bodies. Biochim Biophys Acta 1123:198-206
81. **Beers MF** 1996 Inhibition of cellular processing of surfactant protein C by drugs affecting intracellular pH gradients. J Biol Chem 271:14361-70
82. **Glasser SW, Burhans MS, Korfhagen TR, et al.** 2001 Altered stability of pulmonary surfactant in SP-C-deficient mice. Proc Natl Acad Sci U S A 98:6366-71
83. **Glasser SW, Detmer EA, Ikegami M, Na CL, Stahlman MT, Whitsett JA** 2003 Pneumonitis and emphysema in SP-C gene targeted mice. J Biol Chem 278:14291-8
84. **Mulugeta S, Nguyen V, Russo SJ, Muniswamy M, Beers MF** 2005 A surfactant protein C precursor protein BRICHOS domain mutation causes endoplasmic reticulum stress, proteasome dysfunction, and caspase 3 activation. Am J Respir Cell Mol Biol 32:521-30

85. **Nogee LM, Dunbar AE, 3rd, Wert S, Askin F, Hamvas A, Whitsett JA** 2002 Mutations in the surfactant protein C gene associated with interstitial lung disease. Chest 121:20-21
86. **Nogee LM, Dunbar AE, 3rd, Wert SE, Askin F, Hamvas A, Whitsett JA** 2001 A mutation in the surfactant protein C gene associated with familial interstitial lung disease. N Engl J Med 344:573-9
87. **Nogee LM** 2002 Abnormal expression of surfactant protein C and lung disease. Am J Respir Cell Mol Biol 26:641-4
88. **Thomas AQ, Lane K, Phillips J, 3rd, et al.** 2002 Heterozygosity for a surfactant protein C gene mutation associated with usual interstitial pneumonitis and cellular nonspecific interstitial pneumonitis in one kindred. Am J Respir Crit Care Med 165:1322-8
89. **Hamvas A, Nogee LM, White FV, et al.** 2004 Progressive lung disease and surfactant dysfunction with a deletion in surfactant protein C gene. Am J Respir Cell Mol Biol 30:771-6
90. **Brasch F, Griese M, Tredano M, et al.** 2004 Interstitial lung disease in a baby with a *de novo* mutation in the SFTPC gene. Eur Respir J 24:30-9
91. **Stevens PA, Pettenazzo A, Brasch F, et al.** 2005 Nonspecific interstitial pneumonia, alveolar proteinosis, and abnormal proprotein trafficking resulting from a spontaneous mutation in the surfactant protein C gene. Pediatr Res 57:89-98
92. **Chibbar R, Shih F, Baga M, et al.** 2004 Nonspecific interstitial pneumonia and usual interstitial pneumonia with mutation in surfactant protein C in familial pulmonary fibrosis. Mod Pathol 17:973-80
93. **Bridges JP, Wert SE, Nogee LM, Weaver TE** 2003 Expression of a human surfactant protein C mutation associated with interstitial lung disease disrupts lung development in transgenic mice. J Biol Chem 278:52739-46
94. **Sanchez-Pulido L, Devos D, Valencia A** 2002 BRICHOS: a conserved domain in proteins associated with dementia, respiratory distress and cancer. Trends Biochem Sci 27:329-32
95. **Vidal R, Frangione B, Rostagno A, et al.** 1999 A stop-codon mutation in the BRI gene associated with familial British dementia. Nature 399:776-81
96. **Vidal R, Revesz T, Rostagno A, et al.** 2000 A decamer duplication in the 3' region of the BRI gene originates an amyloid peptide that is associated with dementia in a Danish kindred. Proc Natl Acad Sci U S A 97:4920-5
97. **Hayami T, Shukunami C, Mitsui K, et al.** 1999 Specific loss of chondromodulin-I gene expression in chondrosarcoma and the suppression of tumor angiogenesis and growth by its recombinant protein in vivo. FEBS Lett 458:436-40
98. **Shiozaki K, Nakamori S, Tsujie M, et al.** 2001 Human stomach-specific gene, CA11, is down-regulated in gastric cancer. Int J Oncol 19:701-7
99. **Beers MF, Mulugeta S** 2005 Surfactant protein C biosynthesis and its emerging role in conformational lung disease. Annu Rev Physiol 67:663-96
100. **Johansson H, Nordling, K., and Johansson, J.** 2005 The Brichos domain-containing C-terminal part of pro-surfactant protein C binds to an unfolded poly-val transmembrane segment. manuscript submitted
101. **Pilot-Matias TJ, Kister SE, Fox JL, Kropp K, Glasser SW, Whitsett JA** 1989 Structure and organization of the gene encoding human pulmonary surfactant proteolipid SP-B. DNA 8:75-86
102. **Voorhout WF, Veenendaal T, Haagsman HP, et al.** 1992 Intracellular processing of pulmonary surfactant protein B in an endosomal/lysosomal compartment. Am J Physiol 263:L479-86
103. **O'Reilly MA, Weaver TE, Pilot-Matias TJ, Sarin VK, Gazdar AF, Whitsett JA** 1989 In vitro translation, post-translational processing and secretion of pulmonary surfactant protein B precursors. Biochim Biophys Acta 1011:140-8
104. **Patthy L** 1991 Homology of the precursor of pulmonary surfactant-associated protein SP-B with prosaposin and sulfated glycoprotein 1. J Biol Chem 266:6035-7

105. **Zaltash S, Johansson J** 1998 Secondary structure and limited proteolysis give experimental evidence that the precursor of pulmonary surfactant protein B contains three saposin-like domains. FEBS Lett 423:1-4
106. **Weaver TE** 1998 Synthesis, processing and secretion of surfactant proteins B and C. Biochim Biophys Acta 1408:173-9
107. **Ikegami M, Takabatake N, Weaver TE** 2002 Intersubunit disulfide bridge is not required for the protective role of SP-B against lung inflammation. J Appl Physiol 93:505-11
108. **Johansson J, Curstedt T, Jornvall H** 1991 Surfactant protein B: disulfide bridges, structural properties, and kringle similarities. Biochemistry 30:6917-21
109. **Vandenbussche G, Clercx A, Clercx M, et al.** 1992 Secondary structure and orientation of the surfactant protein SP-B in a lipid environment. A Fourier transform infrared spectroscopy study. Biochemistry 31:9169-76
110. **Tokieda K, Whitsett JA, Clark JC, et al.** 1997 Pulmonary dysfunction in neonatal SP-B-deficient mice. Am J Physiol 273:L875-82
111. **Vorbroker DK, Profitt SA, Nogee LM, Whitsett JA** 1995 Aberrant processing of surfactant protein C in hereditary SP-B deficiency. Am J Physiol 268:L647-56
112. **Melton KR, Nesslein LL, Ikegami M, et al.** 2003 SP-B deficiency causes respiratory failure in adult mice. Am J Physiol Lung Cell Mol Physiol 285:L543-9
113. **Hamvas A, Cole FS, deMello DE, et al.** 1994 Surfactant protein B deficiency: antenatal diagnosis and prospective treatment with surfactant replacement. J Pediatr 125:356-61
114. **Nogee LM, Wert SE, Proffit SA, Hull WM, Whitsett JA** 2000 Allelic heterogeneity in hereditary surfactant protein B (SP-B) deficiency. Am J Respir Crit Care Med 161:973-81
115. **Cole FS, Hamvas A, Rubinstein P, et al.** 2000 Population-based estimates of surfactant protein B deficiency. Pediatrics 105:538-41
116. **Beers MF, Hamvas A, Moxley MA, et al.** 2000 Pulmonary surfactant metabolism in infants lacking surfactant protein B. Am J Respir Cell Mol Biol 22:380-91
117. **deMello DE, Heyman S, Phelps DS, et al.** 1994 Ultrastructure of lung in surfactant protein B deficiency. Am J Respir Cell Mol Biol 11:230-9
118. **Foster CD, Zhang PX, Gonzales LW, Guttentag SH** 2003 In vitro surfactant protein B deficiency inhibits lamellar body formation. Am J Respir Cell Mol Biol 29:259-66
119. **Ballard PL, Nogee LM, Beers MF, et al.** 1995 Partial deficiency of surfactant protein B in an infant with chronic lung disease. Pediatrics 96:1046-52
120. **Dunbar AE, 3rd, Wert SE, Ikegami M, et al.** 2000 Prolonged survival in hereditary surfactant protein B (SP-B) deficiency associated with a novel splicing mutation. Pediatr Res 48:275-82
121. **Klein JM, Thompson MW, Snyder JM, et al.** 1998 Transient surfactant protein B deficiency in a term infant with severe respiratory failure. J Pediatr 132:244-8
122. **Nogee LM** 2004 Genetic mechanisms of surfactant deficiency. Biol Neonate 85:314-8
123. **Robertson B, Halliday HL** 1998 Principles of surfactant replacement. Biochim Biophys Acta 1408:346-61
124. **Possmayer F, Nag K, Rodriguez K, Qanbar R, Schurch S** 2001 Surface activity in vitro: role of surfactant proteins. Comp Biochem Physiol A Mol Integr Physiol 129:209-20
125. **Palmblad M, Johansson J, Robertson B, Curstedt T** 1999 Biophysical activity of an artificial surfactant containing an analogue of surfactant protein (SP)-C and native SP-B. Biochem J 339 381-6
126. **Johansson J, Some M, Linderholm BM, Almlen A, Curstedt T, Robertson B** 2003 A synthetic surfactant based on a poly-Leu SP-C analog and phospholipids: effects on tidal volumes and lung gas volumes in ventilated immature newborn rabbits. J Appl Physiol 95:2055-63

127. **Oosterlaken-Dijksterhuis MA, Haagsman HP, van Golde LM, Demel RA** 1991 Characterization of lipid insertion into monomolecular layers mediated by lung surfactant proteins SP-B and SP-C. Biochemistry 30:10965-71
128. **Qanbar R, Cheng S, Possmayer F, Schurch S** 1996 Role of the palmitoylation of surfactant-associated protein C in surfactant film formation and stability. Am J Physiol 271:L572-80
129. **Nag K, Munro JG, Inchley K, Schurch S, Petersen NO, Possmayer F** 1999 SP-B refining of pulmonary surfactant phospholipid films. Am J Physiol 277:L1179-89
130. **Taneva SG, Keough KM** 1994 Dynamic surface properties of pulmonary surfactant proteins SP-B and SP-C and their mixtures with dipalmitoylphosphatidylcholine. Biochemistry 33:14660-70
131. **Taneva S, Keough KM** 1994 Pulmonary surfactant proteins SP-B and SP-C in spread monolayers at the air-water interface: III. Proteins SP-B plus SP-C with phospholipids in spread monolayers. Biophys J 66:1158-66
132. **Perez-Gil J, Tucker J, Simatos G, Keough KM** 1992 Interfacial adsorption of simple lipid mixtures combined with hydrophobic surfactant protein from pig lung. Biochem Cell Biol 70:332-8
133. **Hawgood S, Ogawa A, Yukitake K, et al.** 1996 Lung function in premature rabbits treated with recombinant human surfactant protein-C. Am J Respir Crit Care Med 154:484-90
134. **Gericke A, Flach CR, Mendelsohn R** 1997 Structure and orientation of lung surfactant SP-C and L-α-dipalmitoylphosphatidylcholine in aqueous monolayers. Biophys J 73:492-9
135. **Clercx A, Vandenbussche G, Curstedt T, Johansson J, Jornvall H, Ruysschaert JM** 1995 Structural and functional importance of the C-terminal part of the pulmonary surfactant polypeptide SP-C. Eur J Biochem 229:465-72
136. **Van Iwaarden JF, Pikaar JC, Storm J, et al.** 1994 Binding of surfactant protein A to the lipid A moiety of bacterial lipopolysaccharides. Biochem J 303:407-11
137. **Kuan SF, Rust K, Crouch E** 1992 Interactions of surfactant protein D with bacterial lipopolysaccharides. Surfactant protein D is an Escherichia coli-binding protein in bronchoalveolar lavage. J Clin Invest 90:97-106
138. **Augusto L, Le Blay K, Auger G, Blanot D, Chaby R** 2001 Interaction of bacterial lipopolysaccharide with mouse surfactant protein C inserted into lipid vesicles. Am J Physiol Lung Cell Mol Physiol 281:776-85
139. **Augusto LA, Synguelakis M, Espinassous Q, Lepoivre M, Johansson J, Chaby R** 2003 Cellular antiendotoxin activities of lung surfactant protein C in lipid vesicles. Am J Respir Crit Care Med 168:335-41
140. **Augusto LA, Synguelakis M, Johansson J, Pedron T, Girard R, Chaby R** 2003 Interaction of pulmonary surfactant protein C with CD14 and lipopolysaccharide. Infect Immun 71:61-7
141. **Johansson J** 2001 Membrane properties and amyloid fibril formation of lung surfactant protein C. Biochem Soc Trans 29:601-6
142. **Gustafsson M, Johansson J** 1998 Structural and functional properties of pulmonary surfactant protein C and related analogs. J Protein Chem 17:540-2
143. **Tjernberg LO, Naslund J, Lindqvist F, et al.** 1996 Arrest of β-amyloid fibril formation by a pentapeptide ligand. J Biol Chem 271:8545-8
144. **Janek K, Rothemund S, Gast K, et al.** 2001 Study of the conformational transition of Aβ(1-42) using D-amino acid replacement analogues. Biochemistry 40:5457-63
145. **Kelly JW** 1997 Amyloid fibril formation and protein misassembly: a structural quest for insights into amyloid and prion diseases. Structure 5:595-600
146. **Fezoui Y, Teplow DB** 2002 Kinetic studies of amyloid β-protein fibril assembly. Differential effects of α-helix stabilization. J Biol Chem 277:36948-54
147. **Stahlman MT, Gray MP, Falconieri MW, Whitsett JA, Weaver TE** 2000 Lamellar body formation in normal and surfactant protein B-deficient fetal mice. Lab Invest 80:395-403

148. **Curstedt T, Jornvall H, Robertson B, Bergman T, Berggren P** 1987 Two hydrophobic low-molecular-mass protein fractions of pulmonary surfactant. Characterization and biophysical activity. Eur J Biochem 168:255-62
149. **Ikegami M, Jobe AH** 1998 Surfactant protein-C in ventilated premature lamb lung. Pediatr Res 44:860-4
150. **Plasencia I, Rivas L, Keough KM, Marsh D, Perez-Gil J** 2004 The N-terminal segment of pulmonary surfactant lipopeptide SP-C has intrinsic propensity to interact with and perturb phospholipid bilayers. Biochem J 377:183-93
151. **Plasencia I, Rivas L, Casals C, Keough KM, Perez-Gil J** 2001 Intrinsic structural differences in the N-terminal segment of pulmonary surfactant protein SP-C from different species. Comp Biochem Physiol A Mol Integr Physiol 129:129-39
152. **Plasencia I, Keough KM, Perez-Gil J** 2005 Interaction of the N-terminal segment of pulmonary surfactant protein SP-C with interfacial phospholipid films. Biochim Biophys Acta 1713:118-28
153. **Karas M, Hillenkamp F** 1988 Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. Anal Chem 60:2299-301
154. **Gobom J, Schuerenberg M, Mueller M, Theiss D, Lehrach H, Nordhoff E** 2001 α-cyano-4-hydroxycinnamic acid affinity sample preparation. A protocol for MALDI-MS peptide analysis in proteomics. Anal Chem 73:434-8
155. **Przybilla L, Brand JD, Yoshimura K, Rader HJ, Mullen K** 2000 MALDI-TOF mass spectrometry of insoluble giant polycyclic aromatic hydrocarbons by a new method of sample preparation. Anal Chem 72:4591-7
156. **Trimpin S, Rouhanipour A, Az R, Rader HJ, Mullen K** 2001 New aspects in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry: a universal solvent-free sample preparation. Rapid Commun Mass Spectrom 15:1364-73
157. **Karas M, Gluckmann M, Schafer J** 2000 Ionization in matrix-assisted laser desorption/ionization: singly charged molecular ions are the lucky survivors. J Mass Spectrom 35:1-12
158. **Karas M, Kruger R** 2003 Ion formation in MALDI: the cluster ionization mechanism. Chem Rev 103:427-40
159. **Chaurand P, Luetzenkirchen F, Spengler B** 1999 Peptide and protein identification by matrix-assisted laser desorption ionization (MALDI) and MALDI-post-source decay time-of-flight mass spectrometry. J Am Soc Mass Spectrom 10:91-103
160. **Bakhoum SF, Agnes GR** 2005 Study of chemistry in droplets with net charge before and after Coulomb explosion: ion-induced nucleation in solution and implications for ion production in an electrospray. Anal Chem 77:3189-97
161. **Hunt DF, Buko AM, Ballard JM, Shabanowitz J, Giordani AB** 1981 Sequence analysis of polypeptides by collision activated dissociation on a triple quadrupole mass spectrometer. Biomed Mass Spectrom 8:397-408
162. **Papayannopoulos IA, Biemann K** 1992 Amino acid sequence of a protease inhibitor isolated from Sarcophaga bullata determined by mass spectrometry. Protein Sci 1:278-88
163. **Roepstorff P, Fohlman J** 1984 Proposal for a common nomenclature for sequence ions in mass spectra of peptides. Biomed Mass Spectrom 11:601
164. **Biemann K** 1988 Contributions of mass spectrometry to peptide and protein structure. Biomed Environ Mass Spectrom 16:99-111
165. **Hvidt A, Linderstrom-Lang K** 1954 Exchange of hydrogen atoms in insulin with deuterium atoms in aqueous solutions. Biochim Biophys Acta 14:574-5
166. **Hoofnagle AN, Resing KA, Ahn NG** 2003 Protein analysis by hydrogen exchange mass spectrometry. Annu Rev Biophys Biomol Struct 32:1-25
167. **Wintrode PL, Friedrich KL, Vierling E, Smith JB, Smith DL** 2003 Solution structure and dynamics of a heat shock protein assembly probed by hydrogen exchange and mass spectrometry. Biochemistry 42:10667-73
168. **Ferraro DM, Lazo ND, Robertson AD** 2004 EX1 hydrogen exchange and protein folding. Biochemistry 43:587-94

169. **Bazzi MD, Woody RW** 1985 Oriented secondary structure in integral membrane proteins. I. Circular dichroism and infrared spectroscopy of cytochrome oxidase in multilamellar films. Biophys J 48:957-66
170. **Johnson WC, Jr.** 1990 Protein secondary structure and circular dichroism: a practical guide. Proteins 7:205-14
171. **Schurch S, Bachofen H, Goerke J, Possmayer F** 1989 A captive bubble method reproduces the in situ behavior of lung surfactant monolayers. J Appl Physiol 67:2389-96
172. **Schurch S, Green FH, Bachofen H** 1998 Formation and structure of surface films: captive bubble surfactometry. Biochim Biophys Acta 1408:180-202