N-Terminally Extended Surfactant Protein (SP) C Isolated from SP-B-Deficient Children Has Reduced Surface Activity and Inhibited Lipopolysaccharide Binding†

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ABSTRACT: In both humans and mice, a deficiency of surfactant protein B (SP-B) is associated with a decreased concentration of mature SP-C and accumulation of a larger SP-C peptide, denoted SP-C_i, which is not observed under normal conditions. Isolation of hydrophobic polypeptides from the lungs of children who died with two different SP-B mutations yielded pure SP-C_i and showed only trace amounts of mature SP-C. 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. The SP-C_i structure determined herein is similar to that of a proposed late intermediate in the processing of proSP-C, suggesting that SP-C_i is the immediate precursor of SP-C. In bronchoalveolar lavage fluid from transgenic mice with a focal deficiency of SP-B, SP-C_i was detected in the biophysically active, large aggregate fraction and was associated with membrane structures that are typical for a large aggregate surfactant. However, unlike SP-C, SP-C_i exhibited a very poor ability to promote phospholipid adsorption, gave high surface tension during cyclic film compression, and did not bind lipopolysaccharide *in vitro*. SP-C_i is thus capable of associating with surfactant lipids, but its 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.

The proteins associated with pulmonary surfactant (SP-A,[1] SP-B, SP-C, and SP-D) promote reduction of the alveolar air−liquid surface tension and participate in lung host defense (*1*, *2*). SP-B and SP-C are strictly hydrophobic and are present in small amounts (1−2 wt %) in surfactant, which make them difficult to isolate and analyze. SP-B is a 17 kDa homodimer which is structurally related to other saposin-like, lipid-binding proteins, including the tumorolytic proteins granulysin/NK-lysin and lysosomal sphingolipid-binding proteins (*3*). SP-B and SP-C individually promote rapid transfer of surfactant phospholipids from an aqueous hypophase to an air−water interface *in vitro*, and SP-B seems to be more efficient in this function (*4*). The physiological importance of SP-B is shown by the fact that newborn infants with inherited deficiency of SP-B, due to mutations in the SP-B gene (*SFTPB*), develop fatal respiratory failure soon after birth (*5*). The most common mutation underlying SP-B deficiency (121ins2) generates a premature stop codon within the sequence encoding the propeptide. Respiratory distress associated with SP-B deficiency is refractory to treatment with exogenous surfactant, and the only effective treatment at present is lung transplantation (*6*, *7*). Similarly, mice deficient in SP-B due to disruption of the SP-B locus die from respiratory distress soon after birth (*8*). Moreover, cessation of SP-B expression in adult mice results in respiratory failure when the concentration of SP-B in bronchoalveolar lavage fluid (BALF) is decreased by 75% (*9*). These data strongly indicate that SP-B fulfils vital functions in the surfactant system. The etiology of respiratory failure in inherited SP-B deficiency is complicated by the fact that SP-B-deficient children and mice show reduced levels of alveolar SP-C and accumulation of a larger peptide which shares immunoreactivity with SP-C (*10*, *11*). The

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[1] Abbreviations: BALF, bronchoalveolar lavage fluid; CID, collision-induced dissociation; ES, electrospray; LPS, lipopolysaccharide; MALDI, matrix-assisted laser desorption ionization; SP, surfactant protein; *SFTPB*, surfactant protein B gene; *SFTPC*, surfactant protein C gene; SP-C_i, processing intermediate of surfactant protein C proprotein.

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isolation and determination of the covalent structure and function of this SP-C peptide (SP-C_i) is the subject of this study.

SP-C is a 4.2 kDa lipopeptide which is uniquely expressed in the alveolar type II cell (*12*). SP-C lacks homologues in the human genome and has an unusually high content of amino acid residues with aliphatic side chains. This feature, together with two palmitoyl groups bound to Cys5 and Cys6, makes SP-C one of the most hydrophobic peptides identified to date (*2*, *13*). The mature SP-C peptide, corresponding to residues 24−58 of a much larger integral membrane precursor protein (proSP-C), is generated via multistep proteolytic cleavage of the C-terminal and N-terminal regions of proSP-C (*14*). Processing of the C-terminal domain is likely initiated in the multivesicular body, and removal of at least part of the N-terminal propeptide is thought to occur in the lamellar body, the intracellular storage granule for surfactant (*15*, *16*).

Mice deficient in SP-C show a variable phenotype, which is dependent on the genetic background; lung structure in some SP-C-deficient mice is virtually identical to that of wild-type mice (*17*), while other strains show progressive inflammatory lung disease (*18*). In contrast to the situation in SP-B deficiency, SP-C-deficient mice exhibit no major disturbance in the other surfactant constituents. A number of different mutations in the SP-C gene (*SFTPC*) have recently been found in association with interstitial lung disease (*19*−*21*). These point mutations are mainly located in the C-terminal region of proSP-C and give rise to single-amino acid replacements or a splicing defect that results in the loss of 37 amino acids encoded by exon 4 (*22*). In these patients, mutant proSP-C is expressed but the protein is apparently not correctly processed and/or transported, leading to intracellular accumulation of proSP-C and very low levels of alveolar SP-C (*19*, *22*). Even though the mutation is present on only one allele, very little proSP-C is processed, suggesting that the mutant form exerts a dominant negative effect on the processing of the nonmutant protein (*22*−*24*). Similarities between the lung pathology in the patients with SP-C mutations and the phenotype of some of the SP-C-deficient mice suggest that a deficiency of alveolar SP-C itself can cause inflammatory lung disease (*18*, *20*). It is also conceivable that accumulation of misprocessed proSP-C in the type II cell contributes to the disease process.

Although the 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 studies *in vitro* (*25*), the knockout mice give little support to the concept that SP-C plays an important role for the reduction of alveolar surface tension *in vivo*. This may reflect functional redundancy between SP-C and SP-B, but it is also possible that SP-C has other functions, which are not directly related to reduction of surface tension. In light of this, SP-C has recently been found to bind to bacterial lipopolysaccharides (LPS) (*26*). Binding of SP-C to LPS involves specific regions of both molecules and modulates LPS−CD14 interactions and cellular responses *in vitro*; however, a physiological role for SP-C−LPS interactions remains to be established (*27*, *28*). Notably, overexpression of SP-B counteracts the effects of LPS in experimental pneumonia in mice (*29*).

In the study presented here, we have isolated SP-C_i from lung tissue of patients with inherited SP-B deficiency, and determined its covalent structure, surface activity, and ability to bind LPS. Moreover, localization of SP-C_i in alveolar surfactant of transgenic mice was analyzed.

EXPERIMENTAL PROCEDURES

Materials. Lung tissue was obtained at the time of transplantation from five children that were homozygous for the 121ins2 mutation (*5*). A small amount of BALF was obtained from one child that was homozygous for the c.479G>T mutation (*30*). Rabbit antirecombinant SP-C antiserum used for detection of mature SP-C was a kind gift from Byk Gulden Chemische Fabrik (Konstanz, Germany). Generation and characterization of a rabbit antibody directed against the recombinant SP-C propeptide (residues 1−20) were previously described (*11*). Alkaline phosphatase-conjugated anti-rabbit IgG was from Sigma (St. Louis, MO). Native SP-C was isolated form porcine lungs using Sephadex LH-60 chromatography and reversed-phase HPLC (*31*, *32*), essentially as described for the isolation of SP-C_i below. SP-C analogues SP-C(Leu) [FGIPSSPVLKRLLILLLLLL-LILLLILGALLMGL (*33*)] and SP-C(LKS) [FGIPSSPVHL-KRLLILKLLLLKILLLKLGALLMGL (*34*)] were synthesized and isolated as described previously. Palmitoylation of Ser residues at positions 5 and 6 of SP-C(Leu) was carried out as described previously (*35*).

Analysis of SP-C_i in Bronchoalveolar Lavage. BALF was obtained from SP-$B_{\Delta C}$ transgenic mice, and large and small aggregate fractions were isolated using sucrose gradient centrifugation (*36*).

Isolation of SP-C_i. Lung tissues, from individual patients or pooled from several patients, were homogenized in 0.1 M KCl and extracted with a chloroform/methanol mixture (2:1) at 4 °C overnight. BALF surfactant was isolated by centrifugation and dissolved in a chloroform/methanol mixture (2:1). The organic phase was recovered, dried, dissolved in a chloroform/methanol/0.1 M HCl mixture [10:10:1 (v/v)], and loaded onto an LH-60 Sephadex column equilibrated in the same solvent system. Fractions eluted from the column were screened by SDS−PAGE and silver staining. Fractions containing SP-C and SP-C_i were recovered and dialyzed (SnakeSkin dialysis tubing, molecular weight cutoff of 3500, Pierce Chemical Co.) against a chloroform/methanol mixture [2:1 (v/v)] overnight at 4 °C to remove HCl, as previously described (*37*). The dialysate was dried down and stored at −80 °C until it was used. For reversed-phase HPLC, samples were dissolved in 95% aqueous ethanol, and insoluble material was removed by centrifugation at 2000*g* for 2 min at room temperature. Reversed-phase separation was carried out using a Vydac C18 column (250 mm × 4.6 mm, 5 μm particles) and solvent combinations optimized for elution of very hydrophobic peptides (*32*). Aqueous methanol (50%) containing 0.1% trifluoroacetic acid (TFA) was used as the initial mobile phase, and elution was performed with a gradient of 0 to 100% 2-propanol containing 0.1% TFA in 8 column volumes, with a flow rate of 0.7 mL/min. Fractions of 1 mL were collected, and peptide-containing fractions were pooled, dried, redissolved in 95% aqueous ethanol, and stored at −20 °C until further use. Circular dichroism spectroscopy shows that SP-C_i dissolved in ethanol or dodecylphosphocholine micelles is predominantly α-helical (data not shown). It is therefore

concluded that the unpolar C-terminal stretch forms a helix, as in SP-C and analogues thereof (*13*), while the structure of the N-terminal part of SP-C_i cannot be determined from the CD spectra only. The secondary structure of SP-C_i was not significantly affected by drying and resolubilization.

Western Blotting. Peptide samples were dissolved in SDS−PAGE sample buffer [0.9 M Tris-HCl (pH 8.45), 24% (v/v) glycerol, 8% (w/v) SDS, 0.015% (v/v) Coomassie blue, and 0.005% (v/v) phenol red], heated for 4 min at 110 °C, and separated using 10 to 20% gradient Tricine gels. Proteins were electrotransferred from the gel to polyvinylidinedifluoride membranes. The membranes were blocked in phosphate-buffered saline (PBS), 0.5% Tween, and 0.05 g/mL nonfat milk, followed by an overnight incubation with antirecombinant SP-C antiserum in a PBS/0.5% Tween solution at room temperature. The membrane was washed and incubated with alkaline phosphatase-conjugated anti-rabbit IgG for 1.5 h in a PBS/0.5% Tween solution at room temperature and visualized in developing buffer containing 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate.

Mass Spectrometry. For matrix-assisted laser desorption ionization (MALDI) mass spectrometry, SP-C_i in 95% ethanol was applied to a 100-well stainless steel plate containing approximately 5 μg of predried α-cyano-4-hydroxycinnamic acid and analyzed using a Voyager De-Pro MALDI TOF (PerSeptive Biosystems) instrument operated in the positive ion mode. Calibration of the *m*/*z* scale was achieved by near-point external calibration, using PF18 (M_r = 2044.43, monoisotopic mass = 2043.09) and insulin (M_r = 5733.58, monoisotopic mass = 5729.60) (*38*).

Electrospray (ES) mass spectra were recorded using a quadrupole time-of-flight instrument (Q-TOF, Micromass). SP-C_i dissolved in 95% aqueous ethanol was sprayed from gold-coated borosilicate capillaries, and argon was used as the collision gas. Deconvolution of the ES data was done using maximum entropy software (Micromass). Ions at *m*/*z* 1365.4, corresponding to $[SP\text{-}C_i + 4H]^{4+}$, were chosen for collision-induced dissociation (CID) experiments. The collision energy was adjusted to obtain optimal fragmentation, and the acquisition range was *m*/*z* 500−2000. Fragment ions derived by peptide bond cleavage are denoted b-type ions, with charge retention on the N-terminal fragment, and y-type ions, with charge retention on the C-terminal fragment (*39*).

Amino Acid Sequence Analysis. SP-C_i was analyzed by Edman degradation in an Applied Biosystems 494 protein sequencer with on-line HPLC for detection of phenylthiohydantoin derivatives.

Surface Activity Measurements. The lipids 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) [7:3 (w/w)] were dissolved in a chloroform/methanol mixture [98:2 (v/v)], and SP-C_i or SP-C dissolved in a chloroform/methanol mixture [1:1 (v/v)] was added. The final peptide concentration was 2% by total mass of phospholipids. The peptide/lipid mixtures were dried under nitrogen and dissolved in saline solution at a final lipid concentration of 10 mg/mL. A captive bubble surfactometer (*40*) was used to record surface tension under quasi-static conditions. Injecting air into the sample chamber containing the peptide/lipid mixtures created the bubble. After an initial adsorption for 5 min, the bubble was compressed stepwise until the surface area could not be reduced further, after which the bubble was expanded stepwise to the original surface area. This process was repeated five times with the same bubble.

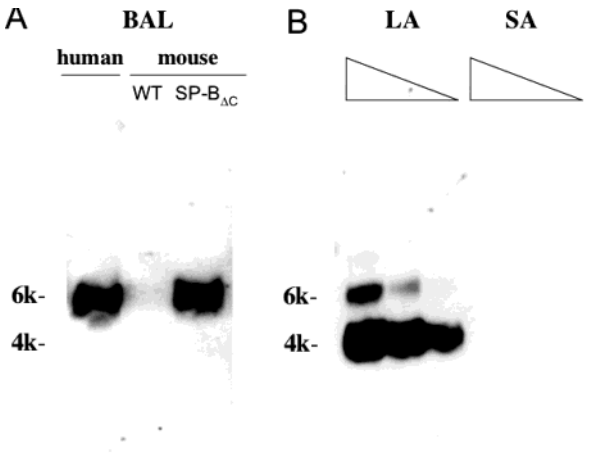


FIGURE 1: SP-C_i associates with large aggregate surfactant. (A) BALF was isolated from an SP-B-deficient patient, wild-type mice, or SP-$B_{\Delta C}$ transgenic mice; aliquots of BALF containing 5, 15, and 15 μg of protein, respectively, were analyzed by SDS−PAGE and Western blotting with antibody directed against the SP-C propeptide. SP-C_i (M_r = 6K) was detected in BALF from the SP-B-deficient patient and SP-$B_{\Delta C}$ transgenic mice but not wild-type (WT) mice. (B) Large aggregate (LA) and small aggregate (SA) surfactant were isolated from BALF of SP-$B_{\Delta C}$ transgenic mice and analyzed by SDS−PAGE and Western blotting with an antibody directed against the SP-C mature peptide. Since the protein could not be detected in the SA fraction, 20, 10, or 5% of each fraction was loaded on the gel. Both SP-C_i (M_r = 6K) and mature SP-C peptide (M_r = 4K) were detected in the LA fraction.

Determination of the Level of Binding to LPS. Binding of tritium-labeled LPS (*Salmonella minnesota* Re-595) to SP-C_i and control peptides was assessed using peptide-coated solvent-resistant microtiter plates (*27*). Briefly, solvent-resistant (polypropylene) microtiter plates were coated with a mixture of tripalmitin and cholesterol (100 μg/well each). Solutions (100 μL) of SP-C_i or control peptides (50−200 pmol) in a chloroform/methanol mixture [1:1 (v/v)] were then added. After evaporation, the films were incubated at room temperature with [^{3}H]LPS Re-595 (350 000 cpm) in the presence or absence of unlabeled LPS (100 μg), in a binding medium (100 μL) containing bovine serum albumin (50 μg) in saline. After being incubated for 3 h, the plates were washed three times with 100 μL of saline, and bound radioactivity was measured. Porcine SP-C and two synthetic SP-C analogues, SP-C(LKS) (*34*) and dipalmitoylated SP-C(Leu) (*33*, *35*), were used as controls in these experiments.

RESULTS

Secretion of SP-C_i in SP-$B_{\Delta C}$ Transgenic Mice. Because it was not possible to obtain sufficient BALF from human SP-B-deficient patients, SP-$B_{\Delta C}$ transgenic mice were used to characterize the association of SP-C_i with different surfactant subfractions (*41*). SP-$B_{\Delta C}$ transgenic mice express a truncated SP-B proprotein in the SP-$B^{-/-}$ background; however, the transgene is not expressed in all type II cells of SP-$B^{-/-}$ mice, resulting in generation of SP-C_i in a small number of cells. SP-C_i (M_r = 6K), similar to that in an SP-B-deficient patient, was detected in BALF from SP-$B_{\Delta C}$ transgenic mice but not wild-type mice (Figure 1A). To

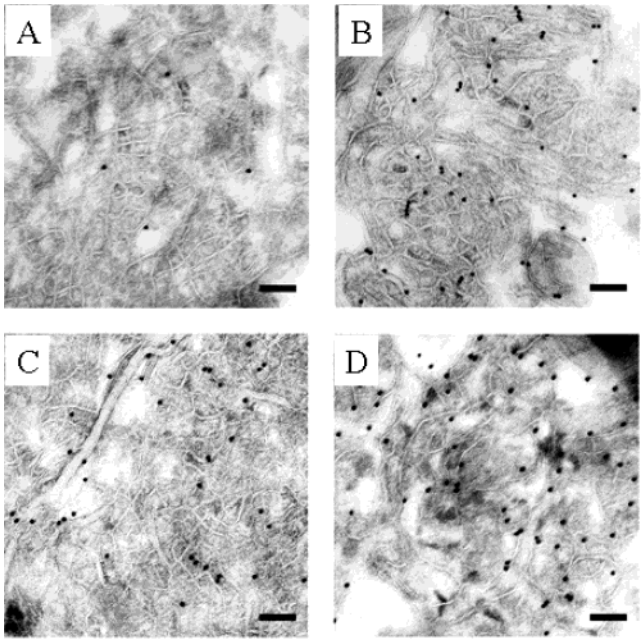


FIGURE 2: SP-C_i associates with characteristic large aggregate membrane forms in the airspace. Immunogold labeling of large aggregate fraction of BALF from (A) wild-type mice and (B) SP-$B_{\Delta C}$ transgenic mice with an antibody directed against the SP-C propeptide. For comparison, the bottom panels show labeling with an anti-SP-B antibody of the large aggregate fraction of BALF from (C) wild-type mice and (D) SP-$B_{\Delta C}$ transgenic mice.

determine if SP-C_i was associated with the biophysically active form of surfactant in the airspaces, the large and small aggregate fractions of surfactant were isolated from BALF. SP-C_i (M_r = 6K) and mature SP-C peptide (M_r = 4K) were detected in the large aggregate fraction of BALF isolated from SP-$B_{\Delta C}$ transgenic mice (Figure 1B). Neither SP-C peptide was detected in the functionally inactive, small aggregate fraction of BALF. The large aggregate fraction was subsequently prepared for cryoultramicrotomy and immunogold labeling with an antibody that detects SP-C_i but not mature SP-C peptide (*11*). SP-C_i was detected in association with membrane structures characteristic of large aggregate surfactant, similar to the labeling pattern for mature SP-B, the major surface active peptide in surfactant (Figure 2). Immunogold labeling of the small aggregate fraction (which is generally considered to be a catabolic form of surfactant) was not detected (data not shown), as predicted by Western analysis. These results indicate that SP-C_i associates with surfactant phospholipids and is secreted into the airspaces with the biophysically active form of surfactant.

Isolation of SP-C_i. SP-C_i was isolated from lung tissues or BALF of patients who were homozygous for the 121ins2 mutation or the c.479G>T mutation. Patients carrying the 121ins2 mutation were treated with bovine replacement surfactant, and their lung tissues consequently contained bovine SP-B and SP-C. Lung tissues from SP-B-deficient human infants were homogenized and extracted with a chloroform/methanol mixture. Chromatography of the organic phase on LH-60 resulted in separation of SP-B (derived from exogenous bovine surfactant treatment) from SP-C; however, SP-C and SP-C_i eluted from the column in the same fractions. Therefore, SP-C_i, SP-C, and lipids were separated by reversed-phase chromatography over a C18 column, using a gradient of 2-propanol running in 50% aqueous methanol

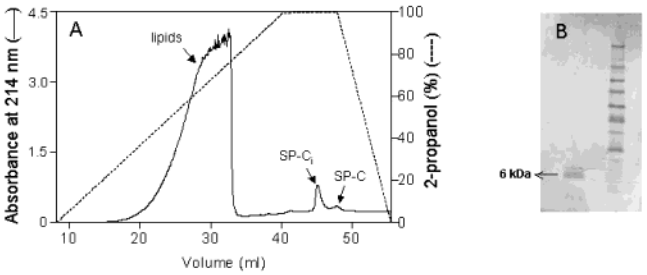


FIGURE 3: Isolation of SP-C_i. (A) Reversed-phase HPLC of the organic extract of lung tissue obtained from an SP-B-deficient child. The main components of the sample were pulmonary lipids that eluted between 50 and 70% 2-propanol. The elution positions of SP-C_i and SP-C are denoted. Note that the SP-C peak mainly contains bovine SP-C, originating from the surfactant preparation used for instillation therapy. (B) Western blot analysis of the SP-C_i HPLC peak. The lane on the right contains molecular mass markers. The arrow identifies SP-C_i, with an estimated molecular mass of 6 kDa.

(Figure 3A). Samples from lung tissues pooled from different patients and from BALF gave essentially the same chromatographic picture. Western blotting of the peptide eluting under the SP-C_i HPLC peak revealed a single band, with an estimated molecular mass of ~6 kDa (Figure 3B). SP-C_i eluted from the reversed-phase column after the lipids and shortly before SP-C, indicating that it is very unpolar but somewhat more polar than SP-C. The SP-C peak contains both human (M_r = 4196) and bovine (M_r = 4057) SP-C (originating from exogenous surfactant therapy) as detected by mass spectrometry (data not shown). As judged from the relative ion intensities, the HPLC peak corresponding to SP-C is dominated by bovine SP-C. This in combination with the fact that only a small amount of SP-C, compared to SP-C_i, was isolated by HPLC (Figure 3A) indicates that only a minor fraction of proSP-C is fully processed to SP-C in SP-B-deficient children.

Covalent Structure of SP-C_i. MALDI mass spectrometry of SP-C_i isolated by HPLC gave a mass of 5457.8 Da, and ES mass spectrometry gave a mass of 5458.0 Da (Figure 4). Amino acid sequence analysis by Edman degradation of SP-C_i yielded the SPPDYSAAPR sequence, which corresponds to residues 12−21 of proSP-C. The calculated molecular mass of a peptide covering positions 12−58 of proSP-C (i.e., ending at the same position as SP-C) with two palmitoyl groups is 5427.2 Da, i.e., 30 Da less than the mass detected by mass spectrometry. In SP-C isolated by the same method used now for isolation of SP-C_i, Met33 was oxidized and the C-terminal Leu was methylated (*42*), together resulting in a mass increase of 30 Da.

CID of SP-C_i (Figure 5) gives rise to three continuous b-ion series, i.e., $b_{31}^{2+}-b_{38}^{2+}$, $b_{24}^{3+}-b_{46}^{3+}$, and $b_{26}^{4+}-b_{46}^{4+}$. In addition, y-ions, predominantly consisting of triply charged ions that cover the region from y_{26} to y_{46}, are found, although no continuous stretch covering more than four ions is detected. This gives a near-complete coverage of the SP-C_i polypeptide (Scheme 1). In particular, the mass difference between y_{29}^{3+} and y_{31}^{3+} shows that Cys17 and Cys18 of SP-C_i are palmitoylated. Likewise, the mass difference between b_{44}^{4+} and b_{45}^{4+} shows that Met45 is oxidized (Figure 5A). Finally, the difference between the mass derived from b_{46}^{4+} (M_r = 5312.4) and the parent ion (M_r = 5458.0) confirms that the C-terminal Leu is methylated. Oxidation of Met45

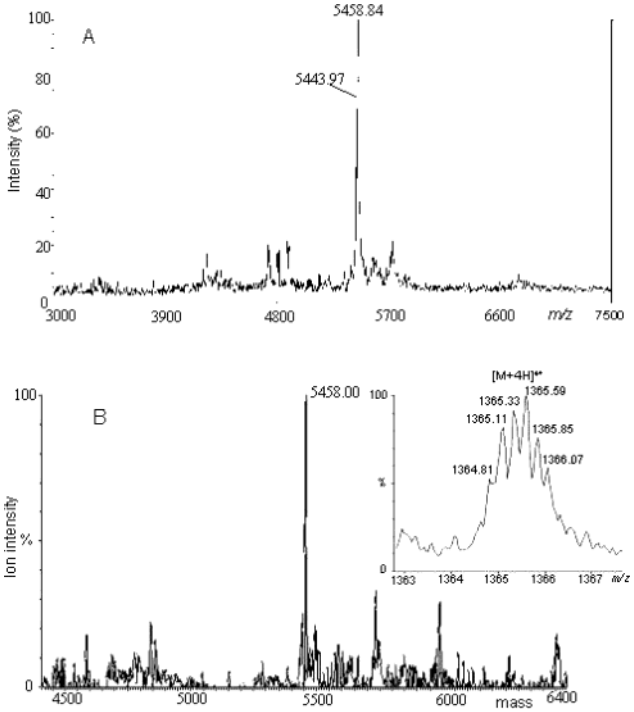


FIGURE 4: Mass of SP-C_i determined by MALDI and ES mass spectrometry. (A) MALDI mass spectrum of SP-C_i showing a peak at $[M + H]^+$ of 5458.8, corresponding to a molecular mass of 5457.8 Da. The peak at $[M + H]^+$ of 5444.0 corresponds to nonoxidized and nonmethylated SP-C_i; see the text for details. (B) Deconvoluted ES mass spectrum of SP-C_i, giving a molecular mass of 5458.0 Da. The inset shows the $[M + 4H]^{4+}$ ion envelope centered at *m*/*z* 1365.4, which was fragmented in the CID experiments.

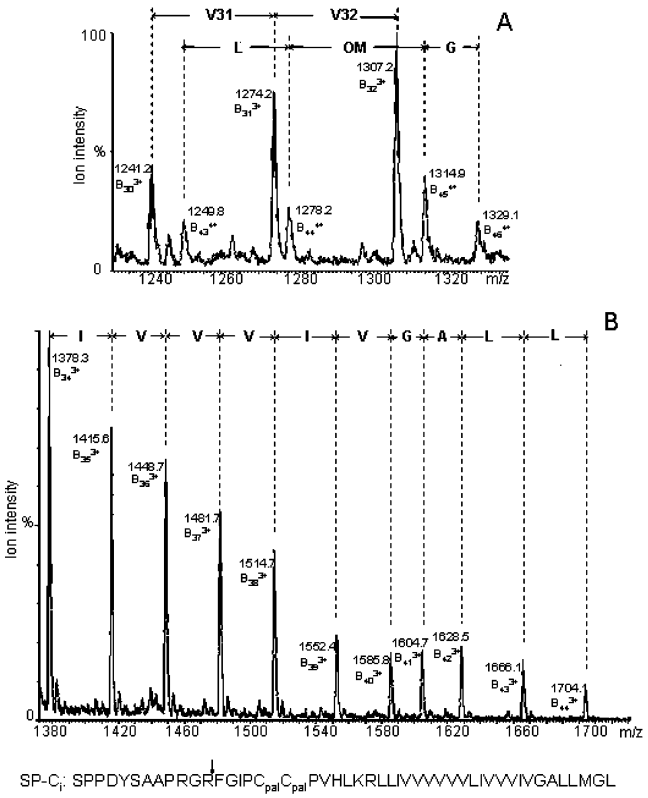


FIGURE 5: CID sequence analysis of SP-C_i. (A) $b_{43}^{4+}-b_{46}^{4+}$ ions yielding the Leu44-oxidized Met45-Gly46 sequence and $b_{30}^{3+}-b_{32}^{3+}$ ions corresponding to residues Val31 and Val32. OM is oxidized Met. (B) $b_{34}^{3+}-b_{44}^{3+}$ ions, which identify the Ile35−Leu44 sequence. All CID fragment ions that are found are summarized in Scheme 1. The covalent structure of SP-C_i is given at the bottom, omitting oxidation of Met45 and methylation of Leu47. The arrow identifies the peptide bond separating the dodecapeptide N-terminal extension unique to SP-C_i from the part corresponding to mature SP-C. Pal represents a palmitoyl group bound to Cys17 and Cys18.

and methylation of Leu47, like in SP-C (*42*), likely occur during the sample workup. In conclusion, mass spectrometry and Edman degradation show unambiguously that SP-C_i consists of 47 amino acids residues, corresponding to an N-terminal dodecapeptide segment followed by the SP-C polypeptide (Figure 5).

Surface Activity of SP-C_i. The surface activity of 2% (w/w) SP-C_i or SP-C in DPPC/POPG vesicles [7:3 (w/w)] was determined with a captive bubble surfactometer. The adsorption characteristics of SP-C_i with phospholipids were similar to those of phospholipids alone, while SP-C promoted the adsorption of phospholipids to the bubble interface (Figure 6A). Inclusion of 2% (w/w) SP-C_i in the phospholipid mixture yielded surface properties during cyclic area compression, which were intermediate between those of SP-C with phospholipids and phospholipids alone (Figure 6B). The results from analysis of SP-C_i isolated from three different pools of lung tissue are summarized in Table 1. Increasing the SP-C_i content to 6% (w/w) did not improve surface activity (data not shown).

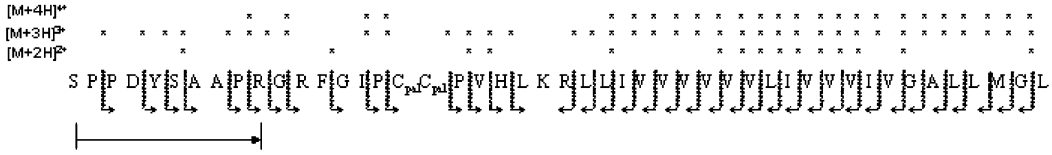
LPS Binding of SP-C_i. The binding of SP-C_i to LPS (*S. minnesota* Re-595) was determined using an assay in which SP-C_i is adsorbed to lipid-coated microtiter plates. This assay can be performed with small amounts of peptide (*27*), and is therefore compatible with the limited amounts of SP-C_i that are available. In a first experiment, the binding of tritium-labeled LPS to 100 pmol of SP-C_i/well or dipalmitoylated SP-C(Leu) was assessed. In this experiment, the level of specific binding was determined by subtracting the level of binding of tritium-labeled LPS in the presence of a large excess of homologous unlabeled LPS (unspecific binding) from the level of total binding. The results show that the level of binding of [^{3}H]LPS to SP-C_i is very low compared to the level of binding to dipalmitoylated SP-C(Leu) (Table 2).

To further compare the LPS binding of SP-C_i with that of mature SP-C and analogues thereof, a second experiment in which the plates were coated with different amounts of peptides was performed. Since for 100 pmol of SP-C_i the level of total binding did not significantly differ from the level of specific binding (Table 2) and limited amounts of SP-C_i are available, binding was analyzed in the absence of unlabeled LPS in this experiment. The results confirm that SP-C_i is devoid of LPS binding activity at concentrations where the LPS binding of porcine SP-C and the two analogues has reached saturation (Figure 7).

DISCUSSION

This study shows, for the first time, that an incompletely processed form of proSP-C found in association with SP-B deficiency (SP-C_i) associates with surfactant phospholipids and is secreted into the airspaces with the large aggregate form of surfactant (Figures 1 and 2), but that it lacks significant surface activity (Figure 6). This indicates that the SP-C_i present in the alveoli does not promote reduction of interfacial surface tension. This, in combination with the

Scheme 1: Survey of Fragment Ions Detected after CID of SP-C_i[a]



[a] Detected b-ions (left-pointing arrows) and y-ions (right-pointing arrows) are denoted with asterisks. The b-ions correspond to peptide fragments starting with Ser1 and ending at the indicated locations, while y-ions start at Leu47 and end at the indicated positions. The arrow below the sequence identifies the residues that were identified by N-terminal Edman degradation.

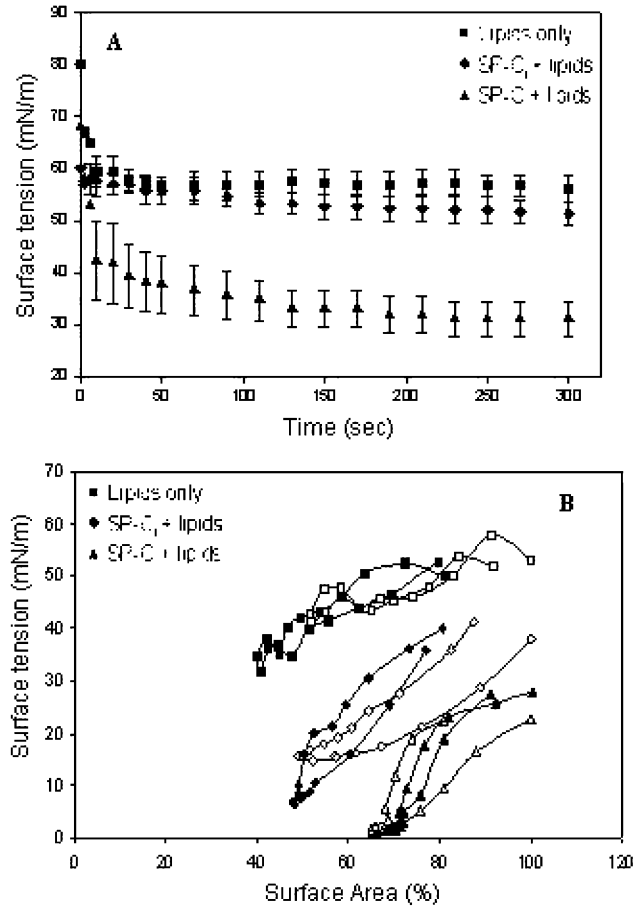


FIGURE 6: Surface activity of SP-C_i. Initial adsorption and surface tension vs surface area as determined from captive bubble quasi-static cycles. In panel A, the surface tension during the initial 5 min following creation of the bubble is shown as mean values and the standard error of the mean for triplicate samples. In panel B, the first cycle for each mixture is depicted using empty symbols, and the fifth cycle is shown with filled symbols. See Table 1 for a summary of cyclic compression data for three SP-C_i isolates.

almost complete absence of mature SP-C, implies that SP-B-deficient children lack active forms of both membrane-associated surfactant proteins. The absence of respiratory dysfunction in SP-C knockout mice (*17*, *18*) suggests that the function of SP-C is not primarily to reduce alveolar surface tension or that its activity can be taken over by other proteins, mainly SP-B. Downregulation of SP-B expression in adult mice leads to respiratory failure, without a major reduction in the amounts of SP-C in BALF, indicating that SP-C does not promote sufficient reduction in alveolar surface tension in the absence of SP-B (*9*). SP-C_i shows no detectable binding to LPS, in sharp contrast to the situation with mature SP-C and analogues thereof (Figure 7). Thus, the presence of the N-terminal dodecapeptide segment in SP-C_i results in strong inhibition of both surface activity and LPS binding, the specific functions so far ascribed to SP-C.

A common denominator for LPS binding of SP-C and analogues thereof is the N-terminal part, and it was concluded that electrostatic forces are important for SP-C−LPS interactions (*27*). The lack of LPS binding to SP-C_i might be caused by the 12-residue extension sterically hindering interactions with the positively charged N-terminal part of SP-C. Similarly, the dodecapeptide extension may inhibit the surface properties of SP-C. It has been proposed that the transmembrane helix is the main feature dictating SP-C−lipid interactions and surface activity (*13*). As the transmembrane part of SP-C is present in SP-C_i, while their surface activities differ, the differences in the N-terminal parts most likely influence surface activity. The 12-residue extension in SP-C_i, containing one acidic residue (Asp4), may partly neutralize the positively charged residues invariably present in the N-terminal part of SP-C. It can be noted that Asp4 in SP-C_i, as well as surrounding residues, is strictly conserved in all species that have been analyzed.

SP-C_i contains an N-terminal dodecapeptide extension, but is otherwise identical to mature SP-C (Figure 5). The C-terminal two-thirds of mature SP-C are mainly composed of a long poly-Val stretch, which folds into an α-helix perfectly suited to span a phospholipid bilayer, while the N-terminal third of SP-C is more polar (*13*). The dodecapeptide specific for SP-C_i, containing three residues with charged side chains and three residues with hydroxyl-containing side chains, is mainly polar and will likely not insert deeply into a phospholipid membrane. SP-C_i thus has a bipartite structure: one C-terminal half which can form a transmembrane α-helix and one N-terminal part which is expected to be localized mainly outside the membrane. It has been suggested that a sequence motif that is important for intracellular targeting is localized in the N-terminal part of proSP-C (*15*, *43*). This motif corresponds approximately to residues 1−7 of SP-C_i, and localization of the N-terminal part of SP-C_i outside the membrane is thus compatible with the accessibility of the proposed targeting motif for cytosolic factors involved in intracellular routing. Proteolytic processing of the N-terminal region of proSP-C occurs in a multistep process (*14*, *15*). Removal of residues 1−11 of the N-terminal propeptide (generating SP-C_i) likely occurs in the cytosol, perhaps during transit of proSP-C to the multivesicular body; the second proteolytic step likely corresponds to cleavage of SP-C_i to SP-C and may occur in the lamellar bodies during or after fusion of the multivesicular body and the lamellar body. It is possible that the low surface activity of SP-C_i, implying altered peptide−lipid interactions, reflects the fact that premature generation of active SP-C could be harmful to the type II cell.

SP-C_i is properly inserted into phospholipid bilayers and secreted to the alveolar space (Figures 1 and 2), which

Table 1: Surface Properties during Captive Bubble Cyclic Area Compression of Mixtures Containing 2% (w/w) SP-C_i or SP-C in DPPC/POPG Vesicles [7:3 (w/w)] or the Phospholipid Mixture Alone[a]

	first cycle			fifth cycle		
peptide	γ_{max} (mN/m)	γ_{min} (mN/m)	Δarea (%)	γ_{max} (mN/m)	γ_{min} (mN/m)	Δarea (%)
SP-C_i	39.7 ± 3.9	15.7 ± 0.9	50.1 ± 3.9	36.6 ± 1.3	12.5 ± 1.0	45.6 ± 5.2
SP-C	31.0 ± 4.0	0.8 ± 0.1	30.2 ± 5.2	35.6 ± 4.7	0.9 ± 0.3	32.6 ± 0.5
phospholipids	53.4 ± 1.3	25.2 ± 8.0	51.7 ± 7.7	52.3 ± 2.2	21.2 ± 5.3	42.1 ± 3.8

[a] The data for the first and fifth cycles are shown as mean values and standard errors of the mean from three different SP-C_i isolates and SP-C with phospholipids and phospholipids alone in triplicate. γ_{max} and γ_{min} are the maximum and minimum surface tension, respectively, and Δarea is the area reduction required to reach γ_{min}.

Table 2: Binding of [^{3}H]LPS Re-595 (350 000 cpm) to Dipalmitoylated SP-C(Leu) and to SP-C_i (100 pmol/well) in the Presence or Absence of 100 μg of Unlabeled LPS[a]

	bound [^{3}H]LPS (cpm)		
peptide	total	unspecific	specific
dipalmitoylated SP-C(Leu)	13860 ± 1447	3236 ± 1157	10634 ± 1069
SP-C_i	3876 ± 748	2278 ± 419	‘598 ± 495

[a] The results are mean values and standard deviations of triplicate determinations.

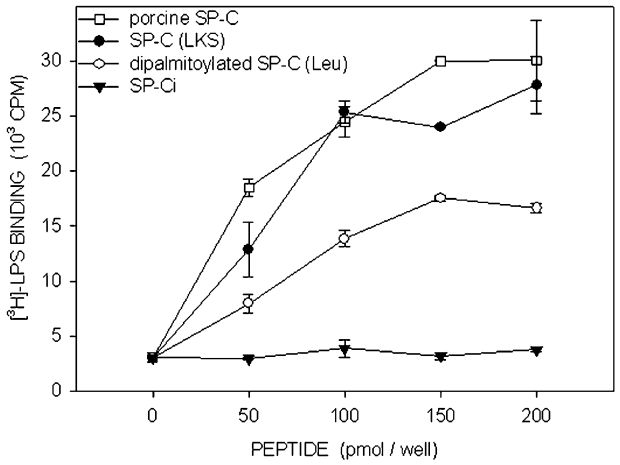


FIGURE 7: SP-C_i does not bind LPS. Binding of LPS to SP-C_i, porcine SP-C, and two analogues of SP-C. Different amounts of the peptides were coated on microtiter plates and the amounts of tritium-labeled LPS (Re-595) determined. The results are mean values and standard deviations of triplicate determinations [porcine SP-C, SP-C(LKS), and dipalmitoylated SP-C(Leu)] or mean values and deviations from the mean of duplicate determinations (SP-C_i).

contrasts with the situation in patients with proSP-C mutations linked to interstitial lung disease, in which little SP-C is secreted into the airspace (*21*). Mutations associated with proSP-C misfolding are localized mainly in the C-terminal proSP-C flanking domain localized in the endoplasmic reticulum lumen, which may be required for folding the SP-C part into an α-helix and/or preventing aggregation. However, the Pro30Leu mutation in the cytosolic part of proSP-C is also associated with interstitial lung disease (*21*), suggesting that this part of proSP-C may also be important for folding. Further work is required to unravel the mechanisms that lead to proSP-C misfolding and aggregation and what role the N- and C-terminal flanking domains play in these processes.

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