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Surfactant protein C precursor is palmitoylated and associates with subcelluiar membranes

Diane K. Vorbroker, Chitta Dey, Timothy E. Weaver and Jeffrey A. Whitsett

Division of Pulmonary Biology, Children's Hospital Research Foundation, Cincinnati, OH (USA)

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Surfactant protein C (SP-C) is a 3.7 kDa, hydrophobic protein that enhances the adsorption of phospholipids in pulmonary surfactant. SP-C is generated by proteolytic processing of a 21 kDa precursor. Murine fetal lung explant cultures and a Chinese hamster ovary cell line expressing recombinant human SP-C gene (CHO/SPC) were used to determine the subcellular location and post-translational modification(s) of proSP-C. After *in vitro* translation, proSP-C of M_r = 21 000 was generated. ProSP-C was associated with canine pancreatic microsomes during co-translation and was partially protected from digestion with proteinase K, supporting the concept that proSP-C enters but does not completely traverse the membrane of the endoplasmic reticulum (ER). Association of proSP-C isoforms of 21 and 26 kDa with intracellular membranes was demonstrated by subcellular fractionation of CHO/SPC cells. Pulse/chase experiments demonstrated that the 21 kDa SP-C proprotein was synthesized first and after 15 min was modified to produce a 26 kDa isoform in CHO/SPC cells or a 24 kDa isoform in murine fetal lung. Both the 21 and 26 kDa proSP-C isoforms were detected after labelling CHO/SPC cells with [^{3}H]palmitic acid. The formation of the 26 kDa proSP-C isoform in CHO/SPC cells and the 24 kDa proSP-C isoform in murine fetal lung was blocked by cerulenin, an inhibitor of fatty acid synthesis. In conclusion, proSP-C is associated with subcellular membranes. ProSP-C is palmitoylated and undergoes additional post-translational modification that is blocked by an inhibitor of fatty acid synthesis.

Introduction

Surfactant protein C (SP-C) is a small hydrophobic protein that is an abundant component of pulmonary surfactant. SP-C enhances the adsorption and surface tension-reducing properties of surfactant phospholipids *in vitro* [1–5]. SP-C is a component of synthetic and bovine surfactant preparations that are utilized *in vivo* as surfactant replacement therapy for infants suffering from Respiratory Distress Syndrome [6].

Although the cDNAs encoding human SP-C predict a precursor of 21 000 daltons [7], in the alveolus, SP-C is a peptide of 33–35 amino acids (3.7 kDa) [8]. The proteolytic events which generate the 3.7 kDa active peptide from the predicted 21 kDa precursor are unknown. In contrast to surfactant proteins SP-A and SP-B, proSP-C does not contain a classical signal sequence to target SP-C to the secretory pathway. Keller et al. [9 recently demonstrated that the region of the 21 kDa SP-C precursor that encodes the hydrophobic active peptide may act as a signal/anchor sequence to integrate fusion proteins into canine pancreatic microsomes *in vitro*. Curstedt and colleagues [10,11] have reported that the 3.7 kDa active SP-C peptide is a true proteolipid, containing covalently linked palmitic acid.

In the present work the routing and post-translational modifications of proSP-C were investigated in Chinese hamster ovary (CHO) cells and murine fetal lung explants. ProSP-C was associated with subcellular membranes and was palmitoylated. After palmitoylation, proSP-C undergoes a further modification which results in an increase in molecular mass. This modification was not observed in the presence of an inhibitor of fatty acid synthesis, suggesting that palmitoylation is required before the modification could occur.

This work was presented in part at the Society for Pediatric Research, New Orleans, May, 1991 [39].

Materials and Methods

Reagents: General

DNA restriction endonucleases and oligonucleotide linkers were from New England Biolabs (Beverly, MA).

Abbreviations: SP, surfactant protein; kDa, kilodalton; ER, endoplasmic reticulum.

Correspondence: J.A. Whitsett, Division of Pulmonary Biology, Children's Hospital Research Foundation, Elland and Bethesda Aves., Cincinnati, OH 45229, USA.

Tissue culture media, Select Amine kit and Hank's Balanced Salt solution were obtained from Gibco BRL (Grand Island, NY). Fetal bovine serum was obtained from ICN Biomedicals, Inc. (Costa Mesa, CA). Cell-free translation reagents were from Promega (Madison, WI) and [^{3}H] were used according to the supplier's specifications. Radioactive reagents were obtained from DuPont New England Nuclear® (Boston, MA) and stored at −20°C until use. Cerulenin, dexamethasone, cycloheximide and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). The pKC4 plasmid was the kind gift of Dr. Douglas Hanahan.

Transfection and maintenance of cell lines

The plasmid used to transfect cells in culture is shown in Fig. 1. The pKC4 plasmid contains the early promoter, poly-adenylation signal, and small t intronic sequences of simian virus 40 (SV40). The human SP-C gene was isolated from a human leukocyte genomic library [12] digested with Bal31 to remove promoter and 3' flanking sequences and inserted into the multiple cloning site of pKC4 using *Xba*I linkers. Human SP-A was isolated from an adult lung cDNA library, flanking regions were removed by digestion with *Bal*31, and the cDNA (785 base pairs in length) was inserted into pKC4 using *Eco*RI linkers.

Chinese hamster ovary (CHO) K-1 cells were transfected with pKC4-hSPC or pKC4-hSPA by the calcium phosphate method [13]. A selectable marker was provided by the plasmid pSV2neo which was co-transfected with the test plasmid in a ratio of 1 to 10. Clones were selected in F-12 media containing 5% fetal bovine serum and 500 μg/ml G418. Media was changed every 3–4 days until well-isolated colonies appeared. Colonies were isolated with cloning rings and tested for expression of SP-C or SP-A by Western blot analysis. Recombinant cell lines are maintained in F-12 media containing 500 μg/ml G418 and 10% fetal bovine serum in 5% CO_2, 95% air at 37°C.

Maintenance of murine fetal lung explants

Mouse strain FVB/N fetuses from day 14–16 of gestation were obtained and whole fetal lung rudiments (referred to in this study as 'fetal lung explants') were cultured on Costar Transwell-Col™ (Cambridge, MA) membranes as described by Glasser et al. [14]. Explant cultures were routinely maintained in Weymouth's media containing 10% fetal bovine serum. For treatment with dexamethasone (Fig. 5, lanes a and b), explant cultures were maintained in Weymouth's media containing 10% carbon-stripped fetal bovine serum as previously described [14].

Antibodies and immunoprecipitation

ProSP-C was expressed as a fusion protein in *Escherichia coli* and was used to immunize rabbits as previously described [15]. SP-A was detected using a rabbit polyclonal antibody against human SP-A purified from human surfactant [16]. A polyclonal antibody generated against chicken gizzard actins that reacts with cytoplasmic and muscle actins was the kind gift of Dr. J. Lessard (Children's Hospital Research Foundation, Cincinnati, OH).

Immunoprecipitation of radioactively-labelled proSP-C and proSP-A was performed as previously described by this laboratory [15]. Incorporation of radioactive label into cellular proteins was determined after precipitation with trichloroacetic acid. The appro-

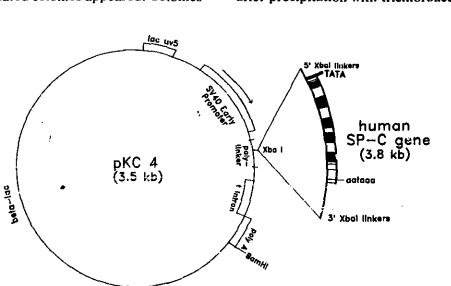

Fig 1. Vector used to create recombinant cell lines expressing surfactant proteins. DNA sequences encoding surfactant proteins were cloned into the multiple cloning site of pKC4 for expression in CHO cells. The entire human SP-C gene was inserted into the multiple cloning site of pKC4 using *Xba*I linkers as shown, to create the CHO/SPC cell line. Exons are shown by boxes with darkened areas representing translated regions of the SP-C gene. Similarly, the cDNA for human surfactant protein A (SP-A) was inserted into the pKC4 plasmid using *Eco*RI linkers (not shown) to create the CHO/SPA cell line.

priate antibody was reacted with cell lysate or *in vitro* translation reaction mixtures overnight at 4°C. Protein G-Sepharose slurry (4 Fast Flow, Pharmacia, LKB Biotechnology, Piscataway, NJ) was added and incubated for 2 h on an oscillating platform at room temperature. Immunoprecipitation complexes were pelleted and washed six times with wash buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.02% SDS, 0.1% Triton X-100), then two times with the same buffer without detergent. Washed immunoprecipitation complexes were boiled for 4 min in sample buffer containing β-mercaptoethanol and proteins were separated by polyacrylamide gel electrophoresis [17]. Proteins were transferred to nitrocellulose paper [18] and the paper exposed to Kodak X-OMAT AR film. For gels containing tritiated samples, nitrocellulose paper was dipped in 2,5-diphenyloxazole prior to exposure to film.

Cell-free translation

Total RNA was isolated from CHO/SPC cells as described by Chirgwin [19]. Poly A^+ RNA was isolated from total RNA on oligo-dT column by the method of Aviv and Leder [20]. SP-A was transcribed from the T7 promoter of pGEM3Z according to the methods of Promega (Madison, WI). Reagents and protocol for *in vitro* translation and co-translation were from Promega (Madison, WI). A typical 50 μl co-translation reaction contained 25 μl wheat germ extract, 1 μl canine microsomes, 40 mM potassium acetate, 1 U RNAsin, 50–100 μCi [^{35}S]cysteine/methionine ([^{35}S]Cys/Met, 45.6 TBq/mmol) and 1–2 μg of poly A^+ RNA. Reactions were stopped with 0.1 mg/ml cycloheximide. To remove non-specific binding of translated proteins to the canine microsomes, microsomes were washed twice by pelleting in a refrigerated microfuge in buffer containing 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 150 mM NaCl. The washed microsomes were aliquoted and were incubated with 50 μg/ml proteinase K in the absence or presence of 1% Triton X-100 for 25 min on ice. Proteinase K reactions were stopped with 1 mM phenylmethylsulfonyl fluoride. ProSP-C and proSP-A were immunoprecipitated as described above.

Metabolic labelling

CHO/SPC or CHO/SPA cultures were prepared for labelling with [^{35}S]cysteine/methionine ([^{35}S]Cys/Met) by pre-incubation for 1 h in labelling medium consisting of Eagle's modified medium lacking cysteine and methionine (Select Amine kit, Gibco) supplemented with 1 mg/ml bovine serum albumin. Medium was replaced with fresh, pre-warmed medium prior to adding label. CHO/SPC cultures were incubated with 100 μCi/ml [^{35}S]Cys/Met (45.6 TBq/mmol) for 4 h during exposure to cerulenin. Pulse/chase studies of CHO/SPC and CHO/SPA cells were carried out as follows: 100 or 400 μCi/ml [^{35}S]Cys/Met was added for the labelling time of 15 min at 37°C, or 2 to 5 min at room temperature. Plates were washed with pre-warmed Hank's Balanced Salts Solution (HBSS) containing 50 μM cycloheximide. This concentration of cycloheximide effectively halts further incorporation of radiolabel into these cells (unpublished observations). HBSS was replaced by pre-warmed Eagle's modified medium, containing 10× concentrations of cysteine (0.24 mg/ml) and methionine (0.15 mg/ml), 1 mg/ml bovine serum albumin and 50 μM cycloheximide, to initiate the 'chase' period.

Prior to labelling with [^{3}H]leucine, CHO/SPC cells were pre-incubated in medium consisting of Eagle's modified medium lacking leucine (Select Amine kit, Gibco) supplemented with 1 mg/ml bovine serum albumin. Cells were labelled with [^{3}H]leucine (100 μCi/ml, 5.7 TBq/mmol) overnight. CHO/SPC cells were labelled overnight with [9,10-^{3}H]palmitic acid (100 μCi/ml,1.4 TBq/mmol) in Dulbecco's Eagle's modified medium containing 5 mM sodium pyruvate (Gibco BRL, Grand Island, NY) and 10 mg/ml fatty acid-free bovine serum albumin. Labelling of CHO/SPC and CHO/SPA cultures was terminated by washing culture dishes with Dulbecco's phosphate-buffered saline (0.9 mM $CaCl_2$, 2.7 mM KCl, 1.1 mM KH_2PO_4, 0.5 mM $MgCl_2$, 138 mM NaCl, 8.1 mM Na_2HPO_4 (pH 7.0)) prior to harvesting.

Murine fetal lung explants were pre-incubated with medium consisting of Eagle's modified medium lacking cysteine and methionine (Select Amine kit, Gibco) supplemented with 1 mg/ml bovine serum albumin. Explants were labelled with 100 μCi/ml [^{35}S]Cys/Met for 4 h at 37°C in 5% CO_2, 95% room air. At the end of labelling, lung rudiments were rinsed with Dulbecco's phosphate-buffered saline and sonicated prior to immunoprecipitation [15].

Inhibition of fatty acid synthesis with cerulenin

Cerulenin was diluted in chloroform, aliquoted into microfuge tubes, dried, and stored at −20°C until use. Dried aliquots were resuspended in 95% ethanol and added directly to culture media. CHO/SPC cells were treated with cerulenin (5–100 μg/ml) in F-12 media containing 5% fetal bovine serum for 2 h prior to radiolabelling. Explant cultures were treated with 0–60 μg/ml cerulenin in Weymouth's medium containing 5% fetal bovine serum for 2 h prior to radiolabelling. Cerulenin-treated explant cultures or cells were radiolabelled with [^{35}S]Cys/Met for 4–6 h in the continued presence of cerulenin.

Subcellular fractionation

Cells were fractionated on a sucrose gradient according to the method of Bamberger and Lane [21]. 10–12 1-ml fractions were taken from the bottom of

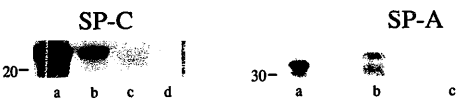

Fig. 2. ProSP-C is inserted into canine pancreatic microsomes. Poly A$^+$ RNA isolated from CHO/SPC cells and SP-A RNA transcribed *in vitro* were translated as described in Materials and Methods. *In vitro* translation was performed in the absence (lanes a) or presence (lanes b) of canine pancreatic microsomes. After co-translation microsomes were treated with proteinase K in the absence (lanes c) or presence (lanes d) of Triton X-100. Approximate molecular masses of immunoprecipitated proteins are indicated in kDa. A faint 20 kDa proSP-C band is visible in lane c, but is absent in lane d. The photo is representative of three separate experiments.

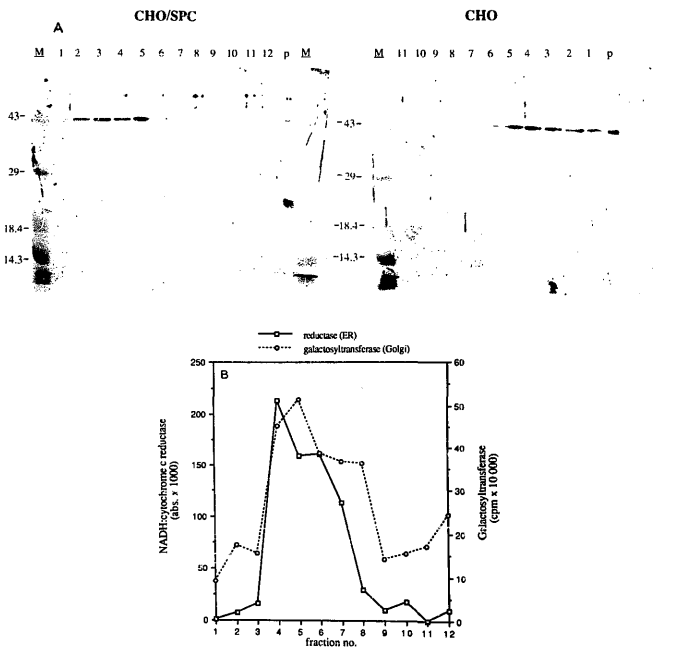

Fig. 3. Subcellular fractionation of CHO and CHO/SPC cells. CHO/SPC cells and CHO K-1 parental cells were fractionated on sucrose gradients according to the method of Bamberger and Lane [21]. Panel A: Fractions were examined for proSP-C by Western blot analysis using antibody R4595. The numbers above the lanes indicate the fraction numbers as taken with a peristaltic pump from the bottom of the sucrose gradient. 'p' represents a low speed pellet containing mostly plasma membrane debris [21]. 'M' represents pre-stained molecular mass markers, as indicated in kDa. Panel B: Fractions from the CHO/SPC gradient were assayed for NADH-cytochrome-c reductase or galactosyltransferase as markers of ER and Golgi, respectively.

the sucrose gradient with a peristaltic pump. Fractions (75–100 μl) were boiled in reducing sample buffer and proteins were separated on 13% polyacrylamide gels [17]. Proteins were transblotted to nitrocellulose and proSP-C detected with antibody R4595 [22,23]. NADH–cytochrome-*c* reductase and galactosyltransferase assays were performed as previously described [24,25].

Results

ProSP-C is associated with subcellular membranes

In vitro translation of poly A^+ mRNA from the CHO/SPC cell line resulted in the production of the 21 kDa precursor, Fig. 2. The 21 kDa translation product consistently co-sedimented with canine pancreatic microsomes after co-translation. Treatment of microsome-associated proSP-C with proteinase K resulted in the generation of a smaller 20 kDa peptide, suggesting that proSP-C was not fully inserted through the membranes and consistent with the recent findings of Keller et al. [9] who demonstrated a shift of 21 kDa proSP-C to 20 kDa after extended co-translation in the absence of proteinase inhibitors. The 32 kDa precursor and 34 kDa glycosylated forms of surfactant protein A (SP-A), a surfactant protein that contains a classic amino-terminal signal sequence, were fully protected from proteinase K digestion under these conditions. Fig. 2.

The subcellular distribution of proSP-C was determined in the CHO/SPC cell line. ProSP-C was detected in fractions from a linear sucrose gradient by Western blot with antibody R4595, Fig. 3A. Two proSP-C isoforms of 21 and 26 kDa were identified in fractions 5–8 of the gradient. The 21 and 26 kDa proSP-C proteins were not detected in the parental CHO cell line. NADH–cytochrome-*c* reductase and galactosyltransferase activity were measured as mark-

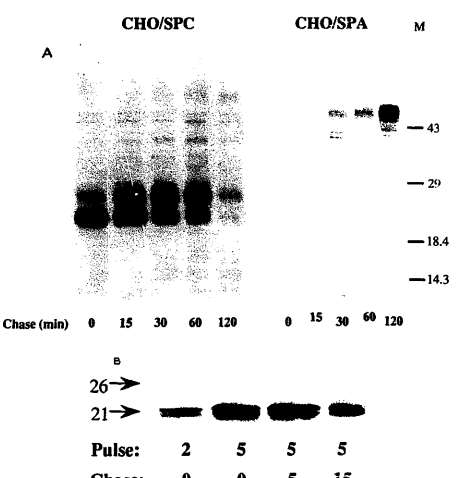

Fig. 4. Time course of the production of the 21 and 26 kDa proSP-C isoforms in CHO cells. Panel A: CHO/SPC and CHO/SPA cells were pulse-labelled with [^{35}S]Cys/Met for 15 min and chased as indicated. Left: proSP-C isoforms of 21 and 26 kDa were immunoprecipitated from CHO/SPC cells with antibody R4595. Lysates were normalized to $1 \cdot 10^6$ trichloroacetic acid-precipitable counts prior to immunoprecipitation and the film was exposed for 7 days. Right: CHO/SPA cells were subject to pulse/chase and lysates were reacted with antibody R4595 parallel to CHO/SPC cells. Lysates were normalized to $2 \cdot 10^6$ trichloroacetic acid-precipitable counts prior to immunoprecipitation. The blot was exposed to X-ray film for 14 days. 'M' represents molecular mass markers, in kDa. Panel B: CHO/SPC cells were labelled with [^{35}S]Cys/Met for 2 or 5 min and chased for the times indicated, in min. The positions of the 21 and 26 kDa proSP-C isoforms are indicated by arrows. The presence of a tight doublet is evidence of alternative splicing of the human SP-C transcript as previously reported [12].

ers of ER and Golgi, respectively, Fig. 3B. Significant levels of these marker enzymes were present in fractions 4–8 indicating the presence of ER and Golgi vesicles in those fractions containing proSP-C isoforms.

Time course of proSP-C biosynthesis in CHO / SPC cells

The time course of the post-translational processing of proSP-C was determined in CHO/SPC cells. The 21 and 26 kDa proSP-C isoforms were detected after labelling with [^{35}S]Cys/Met for 15 min, Fig. 4A. The abundance of both the 21 and 26 kDa isoforms diminished with time. By 240 min of chase the 21 kDa isoform could not be detected and the abundance of the 26 kDa proSP-C isoform was greatly diminished (not shown). In addition to the 21 and 26 kDa precursor isoforms, 14 and 16 kDa forms of SP-C were visible in the chase period from 30 to 120 min after long exposure to film (not shown). The 14 and 16 kDa proteins were not present in immunoprecipitations with antibody R4595 from CHO cells expressing SP-A (CHO/SPA, Fig. 4A) and are likely to represent proteolytically processed forms of SP-C in recombinant CHO/SPC cells. ProSP-C isoforms and proteolytically-processed forms of SP-C were not detected by immunoprecipitation from the media of CHO/SPC cells with antibody R4595 (data not shown).

The early stages of the biosynthesis of proSP-C were determined in CHO/SPC cells using labelling times of 2 and 5 min. Only the 21 kDa SP-C precursor was detected after labelling with [^{35}S]Cys/Met for 2 or 5 min, Fig. 4B. Both the 21 and 26 kDa proSP-C isoforms were detected when cells were labelled for 5 min followed by a 15-min chase in the presence of cycloheximide, Fig. 4B.

Time course of proSP-C synthesis in murine fetal lung explants

Two isoforms of proSP-C were detected in murine fetal lung explant cultures. A 21 kDa precursor (consistent with the known size of murine proSP-C, [26]) and a 24 kDa isoform were immunoprecipitated with antibody R4595, Fig. 5. Both the 21 and 24 kDa isoforms increased in abundance when the explants were cultured with 50 nM dexamethasone. Addition of dexamethasone has been shown to increase SP-C mRNA in murine fetal explants [14].

The time course of the synthesis of proSP-C in murine fetal lung was determined by labelling explants for 5, 15, or 30 min with [^{35}S]Cys/Met. The 21 kDa SP-C precursor was detected after labelling for 5 min, Fig. 5. The 24 kDa proSP-C isoform was detected only after labelling with [^{35}S]Cys/Met for 15 and 30 min. The time course of the appearance of the 24 kDa proSP-C isoform in murine fetal lung is consistent with that of the 26 kDa isoform in the CHO/SPC cell line.

Palmitoylation of proSP-C is required for the formation of the 26 kDa isoform

The 21 and 26 kDa isoforms of proSP-C were radiolabelled with [^{3}H]palmitic acid, Fig. 6. [^{3}H]Palmitic

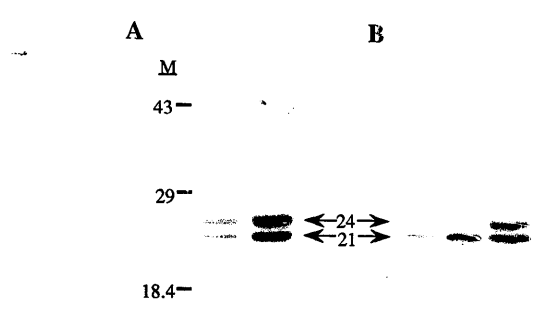

Fig. 5. Detection of two proSP-C isoforms in murine fetal lungs. Murine fetal lung explant cultures were labelled with [^{35}S]Cys/Met and proSP-C was immunoprecipitated with antibody R4595 as described in Materials and Methods. Panel A: Whole lungs from day 16 fetuses (mean weight 622 mg) were cultured for 48 h in the absence (lane a) or presence (lane b) of 50 nM dexamethasone prior to radiolabelling for 4 h. Lysates were normalized to $2 \cdot 10^{6}$ trichloroacetic acid-precipitable counts prior to immunoprecipitation. Panel B: Lungs from day 14 fetuses (mean weight 217 mg) were maintained in culture for 2 days then subject to labelling times of 5, 15, or 30 min, as indicated below the lanes. The position of the 21 and 24 kDa murine proSP-C isoforms are indicated by arrows. 'M' represents molecular mass standards, in kDa.

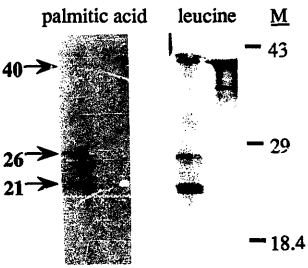

Fig. 6. Labelling of 21 and 26 kDa proSP-C isoforms in CHO/SPC cells with [^{3}H]palmitic acid. CHO/SPC cells were labelled overnight with [^{3}H]palmitic acid or [^{3}H]leucine. Immunoprecipitated proteins were separated by electrophoresis and transferred to nitrocellulose, which was dipped in 2,5-diphenyloxazole prior to exposure to film. [^{3}H]Palmitic acid-labelled proteins were immunoprecipitated with antibody R4595, lane a, or an antibody against chicken gizzard actin that recognizes cytoplasmic and muscle actins, lane b. [^{3}H]Leucine-labelled proteins were immunoprecipitated with antibody R4595, lane c, or anti-actin antibody, lane d. Arrows indicate the position of the 21 and 26 kDa proSP-C isoforms as well at the major actin band (40 kDa). Lanes a and b were exposed to x-ray film for 42 days at −80°C; lanes c and d were exposed to film for 7 days at −80°C. 'M' represents molecular mass markers in kDa.

acid was not incorporated into actins of CHO/SPC cells, demonstrating that [^{3}H]palmitic acid was not being converted to amino acids during the labelling period. Due to the relatively low incorporation of label into proSP-C, pulse chase experiments were not possible using [^{3}H]palmitic acid. The antibiotic cerulenin [27] was therefore utilized to further investigate the palmitoylation of proSP-C. Cerulenin inhibited the formation of the 26 kDa proSP-C isoform in CHO/SPC cells at doses of 20 μg/ml and higher, Fig. 7. High doses of cerulenin also inhibited the synthesis of the novel 24 kDa proSP-C isoform in murine fetal lung explants. Fig. 7. Cerulenin had no effect on the incorporation of [^{35}S]Cys/Met into cellular protein at doses up to 50 μg/ml (not shown).

Discussion

The present work demonstrates that SP-C precursor protein is palmitoylated and is associated with subcellular membranes. Further, proSP-C undergoes an additional post-translational modification occurring up to 15 min after proSP-C is synthesized and synthesis of the larger 24–26 kDa isoforms is blocked by the antibiotic cerulenin, an inhibitor of fatty acid synthesis.

The association of proSP-C with canine pancreatic microsomes and subcellular vesicles suggests that proSP-C contains sequences capable of directing its insertion into the membrane of the endoplasmic reticulum (ER). In contrast to surfactant proteins SP-A and SP-B, proSP-C does not contain an amino-terminal signal sequence to direct its insertion into the ER. The mature SP-C peptide found in surfactant is extremely hydrophobic and is encoded in amino acids 24–60 of the 194 amino acid human SP-C precursor. Several transmembrane proteins contain an internal sequence comprised of hydrophobic amino acids that acts not only as a signal for translocation through the ER membrane, but also as a membrane anchor [28–30]. The mature SP-C peptide portion of proSP-C is a candidate region for such a signal/anchor sequence and has recently been shown to direct insertion of fusion proteins into canine microsomal membranes *in vitro* [9]. The 21 kDa microsome-associated SP-C precursor is processed to 20 kDa after digestion with

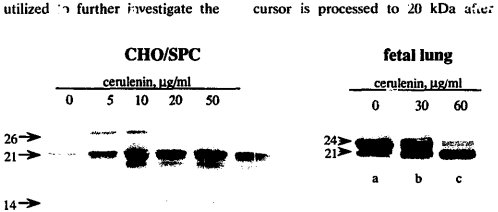

Fig. 7. Effect of cerulenin on the synthesis of proSP-C. CHO/SPC cells or murine fetal lung explants were exposed to the antibiotic cerulenin, an inhibitor of fatty acid synthesis. Cultures were labelled with [^{35}S]Cys/Met during the final 4 h of cerulenin treatment. ProSP-C isoforms were immunoprecipitated from cell lysates with antibody R4595. CHO/SPC cells were exposed to 0–50 μg/ml cerulenin for 6 h (left panel lanes a–e). Poly A$^+$ RNA from CHO/SPC cells was translated *in vitro* and proSP-C was immunoprecipitated with antibody R4595, lane f. The positions of the 21 and 26 kDa human proSP-C isoforms and a 14 kDa proteolytic product are indicated by arrows. Lungs from day 14 fetuses (mean weight 276 mg) were exposed to 0–60 μg/ml cerulenin for 8 h (right panel lanes a–c). The positions of the 21 and 24 kDa murine proSP-C isoforms are indicated by arrows.

proteinase K (Fig. 2), consistent with the findings of Keller et al. [9] who demonstrated the shift of 21 kDa proSP-C to 20 kDa in co-translation reactions in the absence of proteinase inhibitors. The nature of the processing events that occur *in vivo* to produce the 3.7 kDa mature SP-C peptide from the 21 kDa SP-C precursor are unknown at this time.

Both the 21 and 26 kDa isoforms of proSP-C in CHO/SPC cells are labelled with [^{3}H]palmitic acid, demonstrating the palmitoylation of the SP-C precursor. Palmitoylation is characterized as an early post-translational event in which palmitic acid is attached to cysteine or serine residues through a thioester or ester linkage, respectively (reviewed in Refs. 31 and 32). Curstedt et al. [10] have reported the covalent linkage of two palmitic acid residues per 3.7 kDa SP-C peptide molecule isolated from lung lavage. The site of palmitoylation in the human SP-C peptide is a pair of cysteines at positions 5 and 6 (positions 28 and 29 of the precursor), immediately adjacent to the hydrophobic 'signal/anchor' sequence of the mature peptide [10,31]. Although there is no consensus site for palmitoylation, a common feature of most palmitoylated proteins is their association with plasma membranes. Taken together with the evidence that proSP-C is associated with subcellular membranes, these data suggest that SP-C is a transmembrane protein that is anchored in the membrane by a hydrophobic transmembrane region and palmitic acid.

Treatments of CHO/SPC cells and murine fetal lung explants with cerulenin eliminated both the 26 and 24 kDa proSP-C isoforms, respectively. The loss of these higher molecular mass forms with cerulenin treatment and the labelling of the 26 kDa proSP-C isoform in CHO/SPC cells with [^{3}H]palmitic acid suggests that the observed shift in molecular mass requires the addition of palmitic acid. However, the modification that produces the 24 and 26 kDa isoforms is not likely to be palmitic acid alone. First, the 21 and 26 kDa proSP-C isoforms in CHO/SPC cells are labelled with [^{3}H]palmitic acid with equal intensity (Fig. 6). Second, a shift in molecular mass of 3–5 kDa is not likely to be accounted for by the addition of palmitic acid alone, which would contribute 256 daltons per molecule, although it is possible that palmitoylation alters migration in polyacrylamide gels to a greater extent. There are two possible explanations for the loss of the higher molecular mass proSP-C isoforms after treatment with cerulenin. The unidentified post-translational modification may be a fatty acid moiety other than palmitic acid, whose synthesis would also be blocked by cerulenin. Alternatively, the palmitoylation of proSP-C may be a prerequisite to the second post-translational modification of proSP-C. One proposed function of palmitoylation is that it may aid in targetting palmitoylated proteins to the plasma membrane. Ras proteins that are palmitoylated fail to reach the cell surface when palmitoylation is blocked [33,34]. In the absence of palmitoylation, the Vesicular Stomatitis virus (VSV) G protein is properly targetted to virus particles but virus budding is reduced [35–37]. However, for other palmitoylated proteins, such as the transferrin receptor, the loss of palmitoylation has no effect on the routing or function of the peptide [38]. It is unknown at present whether palmitoylation plays a part in the routing of proSP-C. The identification of the second post-translational modification of proSP-C will provide important insight into the synthesis and function of SP-C in the developing lung.

Acknowledgements

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