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Surfactant protein composition of lamellar bodies isolated from rat lung

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Lamellar bodies isolated from rat lung contain all three classes of surfactant proteins, SP-A, SP-B and SP-C, as determined by immunoblot analysis. The amounts of the surfactant proteins present in lamellar bodies, determined by sandwich e.l.i.s.a. (SP-A) and fluorescamine assay (SP-B and SP-C) show that these organelles are highly enriched in the hydrophobic surfactant proteins SP-B and SP-C.

INTRODUCTION

Pulmonary surfactant consists of a mixture of specific lipids and small quantities of unique proteins. Three families of lung-specific apoproteins have been described [1]. SP-A, the most well-characterized of these proteins, is a glycoprotein with an M_r of 26000–38000 under reducing conditions [1,2]. SP-A has been shown to play a role in the formation of tubular myelin in the presence of Ca^{2+} [3,4] and the hydrophobic protein SP-B [4]. SP-A also inhibits secretion and stimulates uptake of phosphatidylcholine by cultured adult rat alveolar type II cells, and thus may be important in the regulation of surfactant homeostasis [5–8]. SP-B and SP-C are very hydrophobic proteins that co-purify with the lipids during extraction of surfactant with organic solvents. SP-B has an M_r of 18000 under non-reducing conditions and an M_r of 5000–8000 under reducing conditions [1,2]. SP-B enhances both the adsorption of phospholipids at an air–liquid interface [9–11] and monolayer enrichment in dipalmitoylphosphatidylcholine [10]. When combined with SP-A, in the presence of Ca^{2+}, the adsorption rate seems to be further enhanced [9,11]. SP-C has an M_r of 5000–8000 under both reducing and non-reducing conditions [1,2]. SP-C may also play a role in enhancing the adsorption of phospholipids at an air–liquid interface [12–14].

Pulmonary surfactant is stored in alveolar type II cells in characteristic multilamellar organelles known as lamellar bodies [15,16]. The goals of this investigation were (1) to demonstrate the presence of the different surfactant proteins in rat lamellar bodies, and (2) to determine the amounts of the individual surfactant proteins present in these organelles. Evidence will be provided that lamellar bodies contain all three classes of surfactant proteins, i.e. SP-A, SP-B and SP-C, and that they are highly enriched in the hydrophobic surfactant proteins SP-B and SP-C.

MATERIALS AND METHODS

Isolation of lamellar bodies and microsomes

Male Wistar rats (180–200 g) were anaesthetized by an intraperitoneal injection of sodium pentobarbital. The lungs were perfused through the pulmonary arteries with 0.25 M-sucrose. Lamellar bodies were isolated from the perfused lungs by the method of Duck-Chong [17], which is based on the principle of upward flotation of lamellar bodies during centrifugation on discontinuous sucrose gradients. Briefly, lung minces were homogenized in 1 M-sucrose (10%, w/v) using a motor-driven Teflon pestle and glass homogenizer, filtered through gauze and layered under a sucrose gradient formed by consecutively layering 0.2–0.8 M-sucrose in 0.1 M increments. Following centrifugation at 80000 *g* at 4 °C for 3 h in a Beckman ultracentrifuge using an SW27 swinging bucket rotor, the lamellar body band was located at the interface of 0.4 M- and 0.5 M-sucrose. The lamellar bodies were collected from the gradient, diluted with an equal volume of distilled water and pelleted by centrifugation at 22000 *g* at 4 °C for 15 min. The lamellar body pellets were resuspended in a small volume of distilled water and stored at −20 °C.

For the isolation of microsomes, lung minces were homogenized (20%, w/v) in 10 mM-Tris/HCl (pH 7.8) containing 250 mM-sucrose and 1 mM-EDTA. After centrifugation at 12000 *g* at 4 °C for 15 min, the pellet was discarded and the microsomes were pelleted by centrifugation at 145000 *g* at 4 °C for 60 min. The microsomes were resuspended in the same buffer (4 mg of protein/ml) and immediately used for the enzyme assays.

Marker enzymes

NADPH:cytochrome *c* reductase was determined according to the method of Phillips & Langdon [18]. We used the method of McMurray [19] to measure contamination of the lamellar body fractions with microsomes by adding variable amounts of microsomal protein to a fixed amount of lamellar body protein (5 μg). The linear regression coefficient of the standard curves was 0.980.

The α-glucosidase activities were measured fluorimetrically in a Perkin–Elmer fluorimeter with a 366 nm excitation filter and a 455 nm emission filter using 4-methylumbelliferyl α-D-glucopyranoside (MeUmb-glu; Sigma Chemical Co., St. Louis, MO, U.S.A.) as fluorescent substrate at a final concentration of 0.44 mM. The reaction mixtures contained 60 μl of sample, 20 μl of MeUmb-glu and 20 μl of buffer. Sodium acetate buffers (100 mM) were used for measurements at acid pH and McIlvaine's buffer [20] for measurements at neutral pH. After 90 min the reactions were terminated by adding 400 μl of 0.3 M-glycine/NaOH (pH 8.6) to the reaction mixtures. To determine the percentage of concanavalin A-negative α-glucosidase activity,

Abbreviations used: SP-A, SP-B and SP-C, surfactant proteins A, B and C; PBS, phosphate-buffered saline; MeUmb-glu, 4-methylumbelliferyl α-D-glucopyranoside.

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75 μl portions of the samples were incubated for 60 min with concanavalin A–Sepharose 4B beads, whereafter the samples were centrifuged (2000 g, 2 min) and the α-glucosidase activity was determined in 60 μl of the supernatants. Concanavalin A–Sepharose 4B beads were first washed and then diluted with 0.5 M-NaCl to a 1:3 (v/v) gel suspension.

All subcellular fractions were sonicated for 6 × 30 s at 0 °C with an MSE ultrasonic disintegrator at 21 kHz and an amplitude of 6 μm peak-to-peak before the enzyme assays.

Isolation of SP-B and SP-C

Lamellar bodies were extracted with butan-1-ol as described by Haagsman *et al.* [21]. Butanol was dried by rotary evaporation and the residue was dissolved in chloroform/methanol/0.1 M-HCl (1:1:0.05, by vol.). Insoluble material was removed by centrifugation. SP-B and SP-C were separated from lipids and from each other by Sephadex LH-60 chromatography (column size 2.5 cm × 80 cm; Pharmacia, Uppsala, Sweden). The column was eluted with the same solvent at a flow rate of 15 ml/h. Fractions of 5 ml were collected, and were analysed by SDS/PAGE and silver staining (silver stain, from Bio-Rad Laboratories, Richmond, CA, U.S.A.). Fractions containing SP-B or SP-C were pooled. The bulk of solvent was removed by rotary evaporation and the remaining water phase was lyophilized. SP-B and SP-C were stored in chloroform/methanol (1:1, v/v) at −20 °C. Protein was estimated by the fluorescamine procedure using BSA as a standard [22]. The protein fractions were analysed for their phospholipid content. The SP-B and SP-C fractions contained 0.8 and 0.1 mol of phospholipid/mol of protein respectively; these values are of the same order as those reported by Curstedt *et al.* [23]. Neither fraction contained detectable amounts of phosphatidylethanolamine.

Electrophoresis and immunoblot analysis

Protein electrophoresis was performed by one-dimensional SDS/PAGE as described by Laemmli [24] or by Tricine SDS/PAGE as described by Schägger & Von Jagow [25]. Some of the samples were reduced with dithiothreitol (25 mM) or with β-mercaptoethanol (5 mM). Gels were stained with Coomassie Brilliant Blue R250 (SP-A) or with silver stain (SP-B and SP-C).

For immunoblot analysis the proteins were transferred electrophoretically from the gels to nitrocellulose filters (Blotting Filter Paper; Bio-Rad). The proteins were detected by immunostaining. Briefly, after blocking non-specific binding with 3% gelatin, the filters were washed, incubated with a primary antibody, washed and incubated with a secondary antibody conjugated with horseradish peroxidase (goat anti-rabbit IgG–HRP, 1:3000; Bio-Rad). Immune complexes were visualized by incubating the filters with peroxidase colour development reagent (Bio-Rad) according to the manufacturer's instructions. The primary antibodies used to detect SP-A and SP-B were rabbit anti-(canine SP-A) (prepared as described below; 1:100) and rabbit anti-SP-B-(1–78)-peptide (1:200) (kindly supplied by Dr. J. A. Whitsett) respectively.

Determination of lamellar body SP-A content

Lamellar body SP-A content was determined by sandwich e.l.i.s.a. using an IgG fraction of a polyclonal antiserum raised in goat against rat SP-A (kindly supplied by Dr. S. L. Young) and a polyclonal antiserum raised in rabbit against canine SP-A which was cross-reactive with rat SP-A. The assay is capable of accurately measuring SP-A at concentrations of 3–50 ng/ml, even in the presence of lipids. Standard curves obtained in this range had a linear regression coefficient of 0.995. Canine SP-A was purified from bronchoalveolar lavage fluid [26] and injected (intracutaneously) into New Zealand rabbits (1 mg/rabbit). Then, at 4, 5 and 6 weeks after the initial immunization, canine SP-A was again injected. The saponin Quil A (Superfos Biosector, Vedbaek, Denmark) was used as an adjuvant. The IgG fraction from immunized goat serum was purified by poly(ethylene glycol) 6000 precipitation [27]. For the e.l.i.s.a., 0.1 ml of goat anti-(rat SP-A) IgG fraction (10 μg/ml in 0.1 M-$NaHCO_3$, pH 9.6) was incubated overnight at 4 °C in wells of untreated microtitre plates (Nunc-Immuno Plate MaxiSorp; Nunc A/S, Roskilde, Denmark). The wells were then allowed to incubate with 1% BSA in phosphate-buffered saline (PBS; 150 mM-NaCl/10 mM-phosphate, pH 7.2) for 30 min at 22 °C. The wells were washed three times with PBS containing 0.05% (w/v) Tween-20. Then 0.1 ml of purified rat SP-A standard or various dilutions of samples in 1% BSA buffered with 5 mM-Tris/HCl (pH 7.4) were added to each well and allowed to incubate for 60 min at 22 °C. The wells were washed and 0.1 ml of rabbit anti-(canine SP-A) serum (1:10000 in 1% BSA/PBS, pH 7.2) was added to each well, followed by incubation for 60 min at 22 °C. After three washes, 0.1 ml of goat anti-rabbit IgG conjugated with horseradish peroxidase (1:10000 in 1% BSA/PBS, pH 7.2; Nordic Immunology, Tilburg, The Netherlands) was added to each well and allowed to incubate for 60 min at 22 °C. The wells were washed and 0.1 ml of substrate solution containing 0.02% (w/v) 3,3′,5,5′-tetramethylbenzidine, 0.003% (v/v) H_2O_2 and 0.1 M-phosphate/citrate buffer (pH 4.0) was added to each well and allowed to incubate for 20 min at 22 °C. The reaction was terminated by the addition of 0.05 ml of 2 M-H_2SO_4 per well. The absorbance at 450 nm was determined with an EASY reader (EAR 400, SLT-Labinstruments, Grodig, Austria).

Lipid analysis

Lipids of the lamellar body fractions were extracted according to the method of Bligh & Dyer [28]. Phospholipids were separated by two-dimensional t.l.c. on silica gel G plates impregnated with boric acid [29] using chloroform/methanol/water/15 M-NH_3 (70:37:4:6, by vol.) and chloroform/methanol/water (13:7:1, by vol.) as developing solvents. After detection by brief exposure to iodine vapour, phospholipids were extracted from the silica gel [28]. Saturated phosphatidylcholine was isolated using the OsO_4 method [30]. Phospholipid phosphorus was estimated according to the method of Bartlett [31].

Protein

Protein was determined by the method of Lowry *et al.* [32] using BSA as a standard. SP-B and SP-C were estimated by the fluorescamine assay [22], also using BSA as a standard.

RESULTS AND DISCUSSION

Properties of the lamellar body fraction

In agreement with Duck-Chong [17], the lamellar body fractions isolated from rat lung consisted of intact lamellar bodies together with large amounts of structures consisting of concentric multilamellated membranes, frequently partly surrounded by a limiting membrane. The lamellar body fractions contained no other cell organelles except for very low amounts of microsomes.

Calculations based on measurements of marker enzymes indicated minimal contamination (less than 1% of protein) of the lamellar body fractions with microsomes. We also tried to determine the concanavalin A-negative α-glucosidase activity in the lamellar body fractions. De Vries *et al.* [33] provided evidence that lamellar bodies isolated from adult human lung contain at least two α-glucosidases with an acid pH optimum: one which

could be precipitated with concanavalin A similar to lung lysosomal α-glucosidase, and another lamellar-body-specific isoenzyme which did not bind to this lectin. The latter enzyme can therefore be used as a lamellar body marker enzyme for studies with human lung. However, lamellar bodies isolated by the method of De Vries *et al.* [33] from rat lung were heavily contaminated with a concanavalin A-negative α-glucosidase with a neutral pH optimum. This was not the case with lamellar bodies isolated from rat lung by the method of Duck-Chong [17]. These preparations contained a total α-glucosidase activity with an acid pH optimum like that reported for human lamellar bodies [33]. However, in contrast with the human lamellar body fractions (results not shown), there was no α-glucosidase activity left in these rat lamellar body fractions after precipitation and removal of concanavalin A-positive activity, except for some (very little) contaminating neutral activity. Therefore acid concanavalin A-negative α-glucosidase cannot be used as a marker enzyme for rat lamellar bodies.

The phospholipid-to-protein ratio in our preparations was 5.4 ± 0.5 μmol/mg (mean ± S.D., $n = 12$). The phosphatidylcholine content of the lamellar bodies was 68.4 ± 8.1 % ($n = 4$) of the total phospholipid phosphorus. Disaturated phosphatidylcholine accounted for 80 % of total phosphatidylcholine of the lamellar body fractions. These results further support the suggestion that these fractions are of lamellar body origin [34–37]. The yield of phospholipid in the purified lamellar body fractions was approx. 0.14 μmol of phospholipid/g of lung, which represents 1 % of total phospholipid/g of lung. Assuming that phosphatidylcholine comprises about 50 % of total lung phospholipid, that disaturated phosphatidylcholine represents approx. 30 % of total lung phosphatidylcholine and that 14 % of disaturated phosphatidylcholine is associated with lamellar bodes (data from ref. [38]), the recovery of lamellar bodies in the purified fraction was estimated to be 25–30 %.

Surfactant protein composition of lamellar bodies

In order to identify SP-A and SP-B in lamellar bodies, immunoblots were performed using antibodies raised against the surfactant proteins SP-A and SP-B. Lamellar bodies contained both surfactant proteins, as indicated on the immunoblots (Fig. 1). Because of the low antigenicity of surfactant protein SP-C, no antibodies raised against SP-C were available. Therefore the presence of SP-C in lamellar bodies was shown in an indirect way. The hydrophobic surfactant proteins were purified from isolated lamellar bodies by Sephadex LH-60 chromatography. The fractions were analysed by SDS/PAGE and silver staining. Under non-reducing conditions fractions containing the SP-B band of M_r 18000 and fractions containing the SP-C band at 5000–8000-M_r were present on the gels (Fig. 2*a*). By immunostaining using antibodies raised against SP-B (not cross-reactive with SP-C), it was shown that the 5000–8000-M_r band indeed represents SP-C and not reduced forms of SP-B (Fig. 2*b*). Other authors have also reported the presence of SP-A [39] and SP-B [39,40] in rat lamellar bodies, but to the best of our knowledge no one so far has shown the presence of SP-C in lamellar bodies.

The amount of SP-A as determined quantitatively by sandwich e.l.i.s.a. was 4 ± 2 μg/μmol of lamellar body phospholipid (Table 1). This is a factor of eight less than the value reported by O'Reilly *et al.* [41]. The reason for this discrepancy is not obvious, but may be at least partially due to the difference in the method of lamellar body isolation. Nevertheless, our result also shows that SP-A is in low abundance among total lamellar body proteins. Assuming a lamellar body recovery of 25–30 %, it can be estimated that 7–8 % of total SP-A in the lung is associated with lamellar bodies.

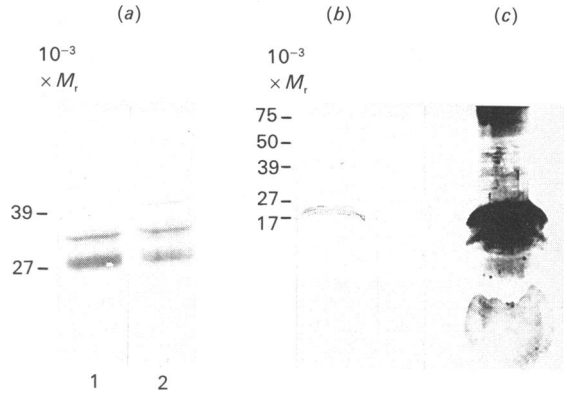

Fig. 1. Identification of surfactant proteins SP-A and SP-B in rat lamellar bodies

Protein electrophoresis was performed by SDS/PAGE in the presence of β-mercaptoethanol (*a*) or by Tricine SDS/PAGE (*b*, *c*). (*a*) Immunostaining with rabbit anti-(canine SP-A) antibodies; lane 1, rat SP-A (0.5 μg) isolated from rat lung lavage; lane 2, total lamellar body protein (25 μg). (*b*) Immunostaining with rabbit anti-SP-B-(1–78)-peptide antibodies; total lamellar body protein (25 μg). (*c*) Total lamellar body protein (25 μg) stained with silver stain.

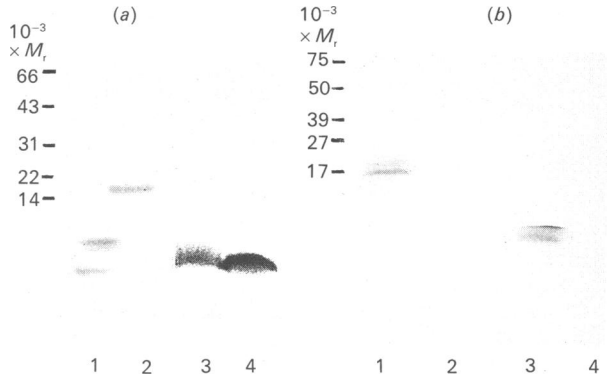

Fig. 2. Indirect identification of surfactant protein SP-C in rat lamellar bodies

Protein electrophoresis was performed by Tricine SDS/PAGE in the absence (non-reducing conditions) or presence (reducing conditions) of β-mercaptoethanol. (*a*) SP-B and SP-C fractions isolated from rat lamellar bodies stained with silver stain. Lane 1, SP-C (0.5 μg) non-reduced; lane 2, SP-B (0.9 μg) non-reduced; lane 3, SP-B (1.0 μg) reduced; lane 4, SP-C (1.0 μg) reduced. (*b*) Immunoblot analysis of SP-B and SP-C fractions isolated from rat lamellar bodies with rabbit anti-SP-B-(1–78)-peptide antibodies. Lane 1, SP-B (0.4 μg) non-reduced; lane 2, SP-C (0.6 μg) non-reduced; lane 3, SP-B (0.4 μg) reduced; lane 4, SP-C (0.6 μg) reduced.

We could not use a sandwich e.l.i.s.a. procedure for SP-B or SP-C. Therefore we determined the amounts of SP-B and SP-C purified from the lamellar bodies by the fluorescamine assay. For SP-B and SP-C isolated from porcine lung lavage, this procedure gives similar estimates to those obtained by quantitative amino acid analysis (results not shown). Because there was variation in the determined amounts of SP-B and SP-C in the different lamellar body fractions, most likely due to variable recovery of the proteins, the values are presented as a range (Table 1). The percentage recoveries of SP-B and SP-C from each purification are not known, but the actual amounts of SP-B and SP-C in the lamellar bodies can only be higher than the ones determined.

Table 1. Amounts of surfactant proteins in lamellar bodies from rat lung determined by sandwich e.l.i.s.a. (SP-A) or fluorescamine assay (SP-B and SP-C)

Results are ranges or means ± S.D. ($n = 4$).

Protein	Amount of protein in lamellar bodies (μg/μmol of phospholipid)	(nmol/μmol of phospholipid)
SP-A	4±2	0.006±0.003
SP-B	40–170	2–9
SP-C	25–140	6–30

Although this method is not really quantitative, the results give a good indication of the amounts of SP-B and SP-C present in lamellar bodies.

When the amounts of SP-A, SP-B and SP-C are calculated as percentages of total lamellar body protein (because SP-B and SP-C are hardly detected by the Lowry procedure, total lamellar body protein is taken to be the amount of lamellar body protein determined by the Lowry procedure plus amounts of the hydrophobic proteins determined by the fluorescamine assay), it appears that lamellar bodies are highly enriched in the hydrophobic surfactant proteins: SP-A constitutes 1%, SP-B 28% and SP-C 22% of total lamellar body protein respectively. SP-B and SP-C are both thought to play a role in the enhancement of the adsorption of phospholipids to an air–liquid interface [9–14]. Therefore the presence of large amounts of the hydrophobic proteins in lamellar bodies may be of importance for the rapid transformation of lamellar bodies to a surfactant monolayer once the lamellar bodies have been secreted into the alveolar subphase.

The same calculations, based on the amounts of surfactant proteins purified from canine alveolar lavage determined by the protein assays (M. A. Oosterlaken-Dijksterhuis, unpublished work), lead to the following percentages: SP-A, 50%, SP-B, 8% and SP-C, 4% of total protein in purified extracellular surfactant. This means that although all three classes of surfactant proteins are present both in lamellar bodies and in extracellular surfactant there is a marked differential distribution of the proteins. This is in agreement with the results of Phizackerley *et al.* [42], who first described the presence of large amounts of hydrophobic proteins in lamellar bodies (about 40% of total lamellar body protein). These authors also reported that the amounts of hydrophobic proteins in extracellular surfactant were much lower (about 13% of total extracellular surfactant protein).

The fact that lamellar bodies are highly enriched in the hydrophobic proteins SP-B and SP-C, whereas extracellular surfactant is highly enriched in SP-A, implies that the turnover of the hydrophobic proteins is somehow independent of the turnover of SP-A. There are two possible explanations for this difference in surfactant protein ratios between lamellar bodies and extracellular surfactant. (1) The amounts of the hydrophobic proteins may be decreased by rapid re-uptake by the type II cell or by rapid degradation of the hydrophobic proteins in the alveolar subphase. (2) The amounts of SP-A may be increased by accumulation of SP-A in the alveolar subphase, either by delayed clearance or by secretion of SP-A into the alveolar subphase by the type II cell (or Clara cell) via a route independent of lamellar bodies. Rapid degradation of the hydrophobic proteins in the alveolar subphase would be in contrast with the idea that the hydrophobic proteins SP-B and SP-C play an essential role in the formation of the surfactant monolayer [9–14]. Thus it is most likely that there is a different recycling pattern for the hydrophobic proteins (SP-B and SP-C) and SP-A. Studies *in vivo* [8] have demonstrated that different forms of surfactant are recycled at different rates and have suggested that there is some specificity in the recycling process. Furthermore, it has been indicated that SP-A not only has a structural and metabolic role in surfactant homeostasis but also is an important factor in the alveolar defence system. SP-A induces activation of alveolar macrophages and enhancement of phagocytosis of these cells [43,44]. Recently it has been reported that synthesis of SP-A can be enhanced by interferon-γ without affecting the production of other surfactant components [45], indeed indicating that SP-A synthesis and secretion may be regulated independently from that of SP-B and SP-C.

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