CMLS, Cell. Mol. Life Sci. 58 (2001) 2098–2107
1420-682X/01/142098-10 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Research Article

Role of phospholipid transfer protein in rabbit lung development

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Received 12 September 2001; accepted 11 October 2001

Abstract. A 36-kDa phospholipid transfer protein ($PLT\text{-}P_R$), which preferentially transfers phosphatidyl choline (PC) compared to phosphatidyl inositol (PI), was purified 827-fold from rabbit lung homogenate. Incorporation of cholesterol in unilamellar vesicles reduced the PC transfer activity of $PLTP_R$. Dipalmitoyl phosphatidyl choline uptake by alveolar type II cells was increased in the presence of the protein, and further enhanced in the presence of surfactant liposomes. However, a decrease in uptake was noted with cholesterol in host membranes. Incorporation of PI into host membranes had a low stimulatory effect on the process. All these effects were more pronounced in adult type II cells compared to premature, term and 3-day-old pups.

Key words. Phospholipid transfer protein; Dipalmitoyl phosphatidyl choline; surfactant; respiratory distress syndrome.

Respiratory distress syndrome (RDS) remains a major cause of morbidity and mortality in neonates. This disease occurs mainly due to the structural immaturity or deficiency of pulmonary surfactant, which is a lipoprotein complex composed of phospholipids (PLs) (80–90%) associated with protein (10–20%) and carbohydrates (1–2%). The phosphatidyl choline (PC) component found mainly as dipalmitoyl phosphatidyl choline (DPPC) accounts for approximately 80% of the total PLs present. The surfactant is synthesized in alveolar type II cells, packaged as lamellar bodies and exocytosed into the alveolar subphase. Thus, it prevents alveolar collapse and transduction at low lung volume by reducing the surface tension at the air-liquid interface [1]. The surfactant is also known to be produced in the fetal lung during the last trimester of gestation and is thus associated with the ability of neonates to establish regular air breathing.

Exogenous surfactant replacement therapy is one of the current modes of treatment for RDS. The surfactants being used clinically are mainly either natural surfactants or synthetic PL mixtures. Designer surfactants or third-generation surfactants (a mixture of PLs and natural or recombinant protein as well as synthetic peptides) are also gaining importance. Furthermore, the presence of proteins like SP-A has been shown to enhance the uptake and reutilization of surfactant in the alveolar subphase [2].

Cytosolic PL transfer protein (PLTP) of alveolar type II cells is known to play an important role in the intercompartmental trafficking of PLs from their site of synthesis [3]. A role for PLTP in lamellar body formation, such as surfactant PL sorting and packaging, has also been suggested by several authors [4, 5]. The PC content is largely controlled by the presence of PC transfer protein (PCTP), which has a preference for the disaturated moeities. Additionally, PC production has also been reported to be modulated by PCTP [6]. PCTP content increased towards the end of gestation in rats and then declined in the post-term period, suggesting an involvement of PCTP in the secretion of lung surfactant [7]. The

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involvement of these PLTPs, especially phosphatidylinositol (PI) transfer protein (PITP), in the signal transduction pathway has also recently come to light [8], and may have an important bearing on the surfactant synthesis and secretion in type II cells. The presence of PLTP in the alveolar subphase [9], although not fully established, has important implications regarding surfactant distribution among various compartments in the alveolar subphase. PLTPs which are involved in the packaging of newly synthesized PLs into maturing lamellar bodies may also play a role in the reutilization of the surfactant components [10]. Although the mechanism of reuptake and the factors required for its regulation are still not well understood, the cumulative information suggests an important role for PLTP in the regulation of intracellular functions in the lung.

Thus, in the present study, a PLTP from rabbit lung homogenate ($PLTP_R$) was purified and an attempt made to investigate its role in the uptake of DPPC by type II cells (isolated at various stages of rabbit lung development) in the presence or absence of surfactant. The outcome of the study may have therapeutic implications for the better reutilization of instilled exogenous surfactant in neonatal RDS.

Materials and methods

Mono Q HR 5/5 column, the commercially available gels (Phast gel TM1 IEF for 3–9) and protein markers were obtained from Pharmacia (Sweden). The Protein PAK 200 SW column was procured from Nihon Waters (Japan). DPPC, PI, and phosphatidyl serine (PS) were obtained from Sigma. Other chemicals and reagents used in the study were of analytical grade.

Isolation and purification of $PLTP_R$

$PLTP_R$ was isolated and purified by the modified methods of Tsao et al. [11]. Briefly, a 25% homogenate of rabbit lung prepared in ice-cold buffer [0.01 M Tris/HCl (pH 7.4) containing 0.33 M sucrose, 5 mM β-mercaptoethanol, 1 mM EDTA, 0.1 mM phenylmethyl sulphonyl fluoride (PMSF) and 10 µg/ml pepstatin A] was subjected to differential centrifugation. The pH of the supernatant (cytosol) was adjusted to 5.6 and allowed to stir (4 °C, 30 min). The precipitated material was removed by centrifugation (6600 g, 10 min). The supernatant (fraction M) was titrated back to pH 7.4 and then subjected to sequential fractionation with different percentage (30, 50 and 70%) saturations of ammonium sulphate [$(NH_4)_2SO_4$] according to Dixon's nomogram [12]. The pellet obtained after 70% saturated $(NH_4)_2SO_4$ precipitation revealed the PL transfer activity. Subsequently, it was suspended in buffer A [0.01 M Tris/HCl (pH 7.4) containing 0.15 M NaCl, 5 mM β-mercaptoethanol and 1 mM ethylene diamine tetra-acetic acid (EDTA)] and fractionated by gel filtration chromatography on a Sephadex G-75 column (2.5 × 82 cm) previously equilibrated with the same buffer at a flow rate of 12 ml/h. Elution was achieved with buffer A. Peak 3 corresponding to pooled fractions (61–63) with the maximum PL transfer activity was concentrated by Amicon ultrafiltration with a UM membrane (10-kDa cut-off). The concentrated sample was subjected to a DEAE-cellulose column (1 × 10 cm) preequilibrated with buffer B [0.01 M Tris/HCl (pH 7.8), 1 mM PMSF]. The column was washed with the same buffer to remove the unbound proteins. The bound proteins were eluted out using a linear gradient of 0–0.25 M NaCl in buffer B. Pooled peak 4 corresponding to fractions 47–50, eluted at 104–111 mM NaCl, with the maximum PL transfer activity was concentrated and further fractionated by anion exchange chromatography on a Mono Q HR 5/5 column in the FPLC system, using the same conditions as for DEAE cellulose chromatography. Peak 2 containing the semipurified $PLTP_R$ was eluted at 182 mM NaCl, desalted, concentrated and finally purified on a gel filtration column (Protein Pak 200 SW, 0.8 × 30 cm) in the FPLC system. Preequilibration of the column was performed with buffer A containing 0.02% sodium azide and 1 mM PMSF at a flow rate of 1 ml/min and elution was achieved with the same buffer. Peak 2 corresponding to the fractions eluted at 8.8–10.8 min showing maximum PL transfer activity was collected, desalted, and concentrated. The $PLTP_R$ containing fractions after each fractionation step were characterized by different analytical methods.

Analytical methods

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, 12.5%) was performed as described by Laemmli [13]. Protein dissociation was carried out by heating at 100 °C for 5 min in Laemmli sample buffer in the presence of β-mercaptoethanol. Protein markers used were bovine serum albumin (BSA, 67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and lysozyme (14.3 kDa). Mobility was measured relative to bromophenol blue. The gels were fixed in 10% trichloroacetic acid (TCA) overnight and stained for protein with Coomassie blue. Analytical isoelectrophoresis was performed in the phast system (Pharmacia, Sweden) on commercially available gels (Phast gel TM1 IEF 3–9), according to the manufacturer's instructions. The gel was run at 2000 V, 2.5 mA and 3.5 W at 15 °C for 75 Vh for prefocusing. Subsequently, the samples were applied to the gel at 200 V, 2.5 mA and 3.5 W at 15 °C for 15 Vh. Finally, the gel was run at 2000 V, 2.5 mA, and 3.5 W at 15 °C for 410 Vh. Amyloglucosidase (pI 3.50), methyl red (pI 3.75), soybean trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.20), bovine carbonic anhydrase B (pI 5.85), human carbonic anhydrase B

(pI 6.55), horse acidic (pI 6.85) and basic (pI 7.35) myoglobin, lentil acidic (pI 8.15), middle (pI 8.45) and basic (pI 8.65) lectin and trypsinogen (pI 9.30) were used as standards.

Determination of protein

Proteins were estimated by the method of Lowry et al. [14] using BSA as standard.

Preparation of liposomes

Small unilamellar vesicles (ULVs) were prepared freshly as described by Gumbhir et al. [15]. Briefly, egg yolk PC (2 µmol), butylated hydroxytoluene (0.2 µmol) as an antioxidant and [^{14}C]-DPPC were dissolved in diethyl ether in a round-bottomed flask. The lipid mixture was flushed with N_2 and evaporated to dryness in a rotary evaporator. This was redissolved in diethyl ether and subsequent evaporation resulted in a thin lipid film, which was further hydrated in 0.05 M Tris/HCl (pH 7.4) containing 0.25 M sucrose and 0.02% sodium azide. The suspension was briefly shaken on a vortex mixer and kept for 2 h at room temperature for swelling. The milky suspension was sonicated with a probe-type sonicator with a microtip (MSE model Soniprep 150) for 30 min with a 20- to 25-s cooling period between each sonication in an ice-water environment. The lipid mixture was centrifuged for 1 h at 140 000 g. The poorly dispersed lipids and titanium metal emanating from the sonicator probe were pelleted, while the slightly transluscent supernatant was used as ULVs for further experiments. This procedure was also used in the preparation of unilamellar liposomes consisting of the following lipids in different percentage mole ratios instead of PC alone: PC and cholesterol (50:50, 90:10), PC and PI (50:50), and as PC and PS (50:50).

Preparation of surfactant liposomes

Liposomes were freshly prepared using the method of Ikegami et al. [16]. An aliquot of the extracted surfactant lipids was dried under nitrogen in a rotary evaporator and hydrated in 5 mM HEPES buffer (pH 7.4) containing 1 M NaCl for 1 h at room temperature. The mixture was sonicated (Soniprep 150) for 5 min with a 20- to 25-s cooling period between each sonication at a 50% energy output of the sonicator and in an ice-water environment. The sonicate was centrifuged at 27 000 g for 20 min. The supernatant was collected and passed repeatedly through a 27-gauze needle. The transluscent supernatant containing the liposomes prepared from the surfactant lipids was used for further experiments.

Estimation of surfactant lipids

Total lipids, total PL phosphorus as well as total cholesterol were estimated by the method of Friengs and Dunn [17], Marinetti [18] and Zak [19], respectively.

Isolation of mitochondria

Rabbit lung mitochondria were isolated from a 25% (w/v) homogenate of the tissue in 0.01 M Tris/HCl (pH 7.4) containing 0.25 M sucrose and 1 mM EDTA [20]. The mitochondrial pellet obtained after differential centrifugation was washed and used for further experiments.

PL transfer assay

PL transfer activities were measured as described by Van Golde et al. [21]. ULVs (0.2 µmol of PLs) radiolabelled with [^{14}C]-PC were incubated with rabbit lung mitochondria (0.5 mg protein) and an appropriate concentration of transfer protein in a shaking water bath at 37 °C for 1 h. The transfer reaction was terminated by addition of chilled buffer [0.01 M Tris/HCl (pH 7.4) containing 0.25 M sucrose] and centrifuged (10 000 g, 5 min) immediately. The mitochondrial pellet was washed and subsequently dissolved in Triton X-100 (0.2 ml, 1% w/v). Radioactivity was assayed in Bray's scintillation fluid (2 ml). Blank incubations (without active protein) were also performed simultaneously. The percentage of PL transferred was calculated as:

$$\frac{\text{counts in the mitochondrial pellet (acceptor)} \times 100}{\text{total counts (donor)}}$$

Animal studies

Adult pregnant rabbits (New Zealand White Strain) were obtained from the Central Animal House of the Institute (PGIMER, Chandigarh, India). The animals were fed standard pellet diet and water ad libitum and housed in individual cages. Premature rabbit fetuses were removed on the 26th day of gestation. Term fetuses (0 days old) and post-natal rabbit pups (3 days old) were also used for a number of experiments.

Isolation of IgG from rabbit serum

IgG was purified from rabbit serum by affinity chromatography on a Protein A-Sepharose CL-4B column [22].

Isolation of alveolar epithelial type II cells from fetal and neonatal lungs

Alveolar type II cells from young adult rabbits, premature rabbit fetuses as well as those from lungs of term and 3-day-old rabbit pups were isolated as described by Dobbs [23]. Cell viability and purity were assessed by the method of Dobbs [23].

Uptake of DPPC by alveolar type II cells

Type II cells (isolated from lungs of four different sets of animals) were incubated for 60 min [24] with PL transfer activity-containing material (obtained after each fractionation step) in the presence and absence of natural surfactant liposomes. In another set of experiments, the

type II cells were incubated in the presence of purified $PLTP_R$ along with liposomes of different PL composition. Type II cells in the absence of $PLTP_R$/the surfactant liposomes/liposomes of different PL composition served as the controls.

Statistical analysis

The data on surfactant composition were expressed as mean ± SD of four to six independent observations as analysed in triplicate. However, comparison of the PL transfer assay system of fetal and adult type II cells in the absence and presence of surfactant liposomes was statistically analysed using Student's t test to establish the validity of the investigation [25].

Results

The elution profiles of the Sephadex G-75 column, the anion exchange columns of DEAE-cellulose as well as the Mono Q and the Protein Pak 200 SW column [used for the sequential fractionation of $PLTP_R$-containing material (obtained after 70% saturated $(NH_4)_2SO_4$ precipitation)] are shown in figures 1–4. Analysis of the protein fractions (monitored at 280 nm) corresponding to peaks P_3 (peak 3 of Sephadex G75 corresponding to fractions 61–63), Q_4 (peak 4 of DEAE-cellulose corresponding to fractions 47–50 eluted with 104–211 mM NaCl), T_2 (peak 2 of Mono Q eluted with 182 mM NaCl) and R_2 (peak 2 of Protein Pak 200 SW corresponding to fractions eluted at 8.8–10.8 min) with the maximum PL transfer activity is shown in SDS-PAGE (fig. 5). The peak P_3 revealed one major and three minor bands (lane 4), Q_4 showed three major along with several minor bands (lane 5). T_2 gave two major as well as two minor bands (lane 6), while R_2 ($PLTP_R$) revealed a single band of Mr 36 kDa (lane 7). The molecular weight of $PLTP_R$ was determined from the plot of relative electrophoretic mobility of the marker proteins against their corresponding molecular weights. Isoelectrofocussing of $PLTP_R$ on a polyacrylamide gel also revealed a single band of pI 6.8 (fig. 6) as determined from the plot of pI of the standards against their respective distances from the anode. $PLTP_R$ was purified 827-fold in terms of its PL transfer activity (table 1). The yield was 57 µg from 957.6 mg of crude material.

PL transfer specificity of $PLTP_R$ as determined by its ability to transfer different PLs (PC and PI) from ULVs to rabbit lung mitochondria is shown in table 2. $PLTP_R$ showed a preference for PC over PI transfer. The changes in the PL transfer activity of $PLTP_R$ following alteration of the PL composition of the donor membrane are given

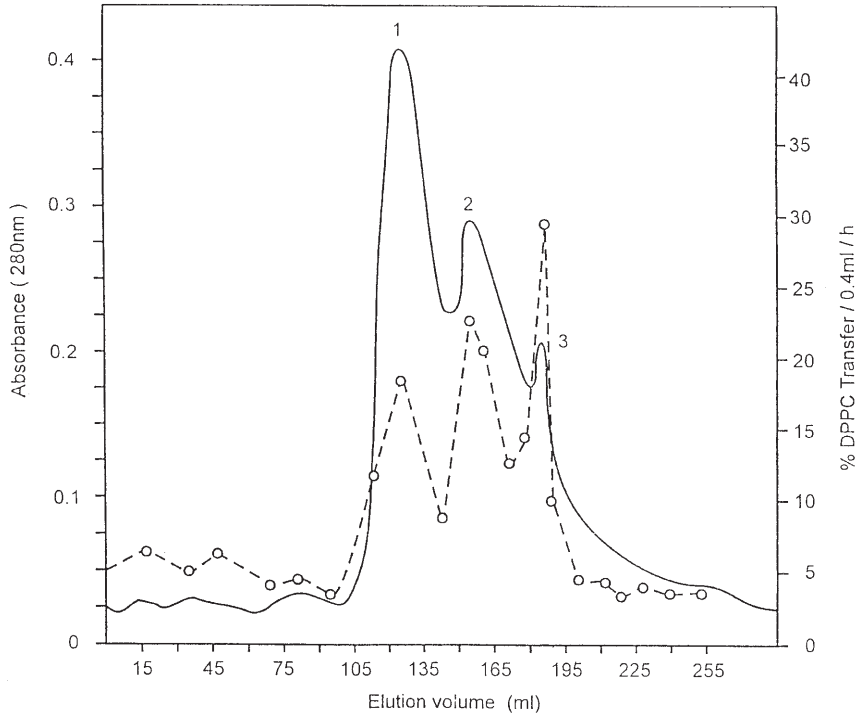

Figure 1. Elution profile of Sephadex G-75 column. The 70% saturated $(NH_4)_2SO_4$ precipitated material was applied to a Sephadex G-75 column (2.5 × 82 cm) preequilibrated with buffer A [0.01 M Tris/HCl (pH7.4)/0.15 M NaCl/5 mM β-mercaptoethanol/1 mM EDTA]. Chromatography was conducted with the same buffer at a flow rate of 12 ml/h. The absorbance of the fractions was monitored at 280 nm (——) for protein. Fractions of 1.5 ml were collected and each fraction was tested for PL activity. Lipid transfer activity is represented as % DPPC transfer/0.4 ml per hour (– – – –).

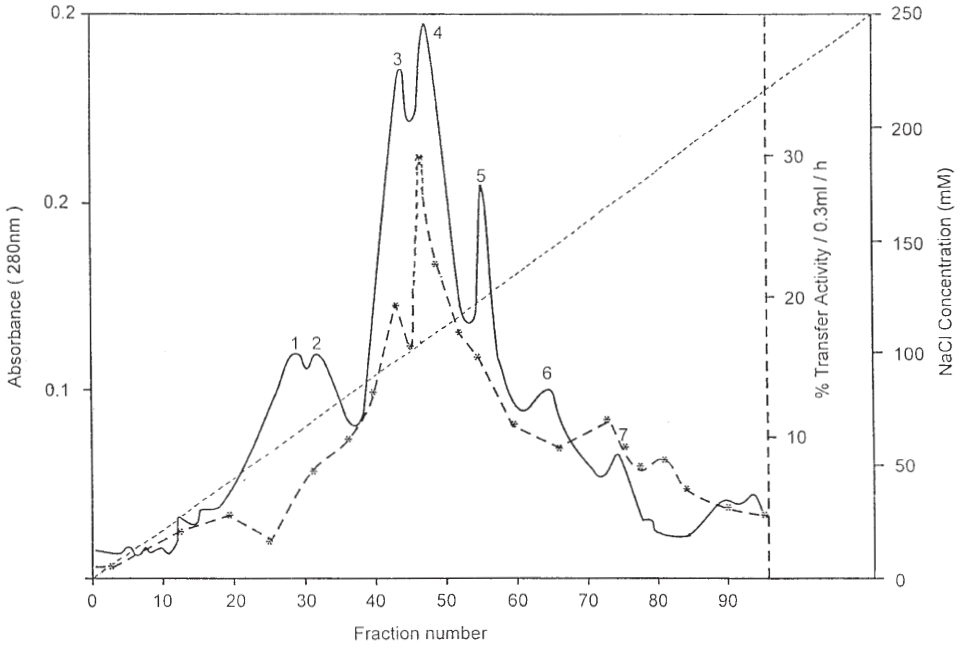

Figure 2. DEAE-cellulose column chromatography. The Sephadex G-75 column-eluted fractions with maximum PL transfer activity were pooled, concentrated and subjected to a DEAE-cellulose column (1 × 10 cm) preequilibrated with buffer B [0.01 M Tris/HCl (pH 7.8)/ 1 mM PMSF] at a flow rate of 16 ml/h. The column was washed with the same buffer to remove the unbound protein and the bound proteins were eluted out using a linear gradient of 0–0.25 M NaCl in buffer B. Each fraction was monitored for protein at 280 nm (——) and assessed for lipid transfer activity (– – – –).

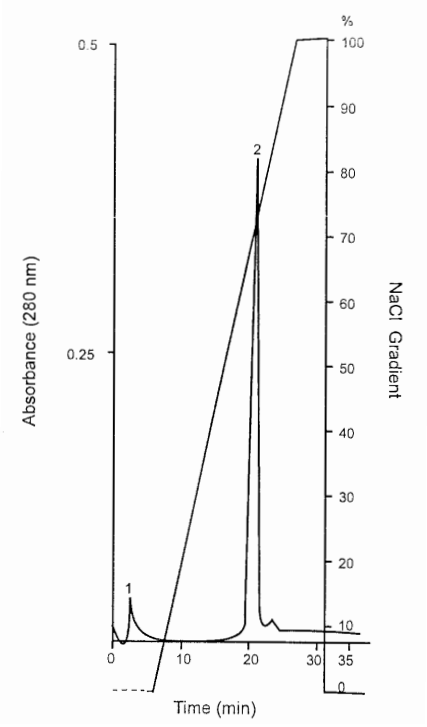

Figure 3. Elution profile of Mono Q column. The DEAE-cellulose column-eluted fractions with maximum PL transfer activity were pooled, concentrated and applied on a Mono Q (HR 5/5) column in the FPLC system. The column was preequilibrated with buffer B at a flow rate of 0.5 ml/min and elution was achieved with a linear gradient of 0–0.25 M NaCl in buffer B. The fractions were monitored for protein at 280 nm and checked for PL activity.

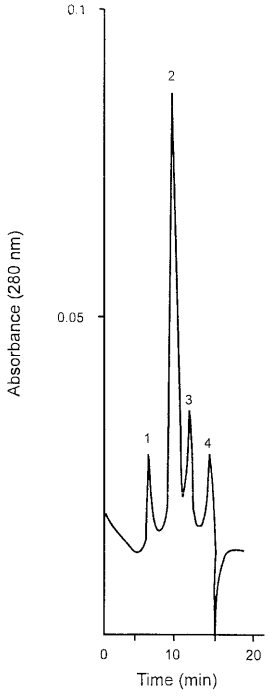

Figure 4. Elution profile of Protein Pak 200 SW gel filtration column. Fraction 2 of the Mono Q column with maximum PL transfer activity was subjected to the gel filtration column (8 × 30 cm) previously equilibrated with buffer A containing 0.02% sodium azide and 1 mM PMSF at a flow rate of 1 ml/min. Elution was achieved with the same buffer. All the fractions were monitored for protein at 280 nm.

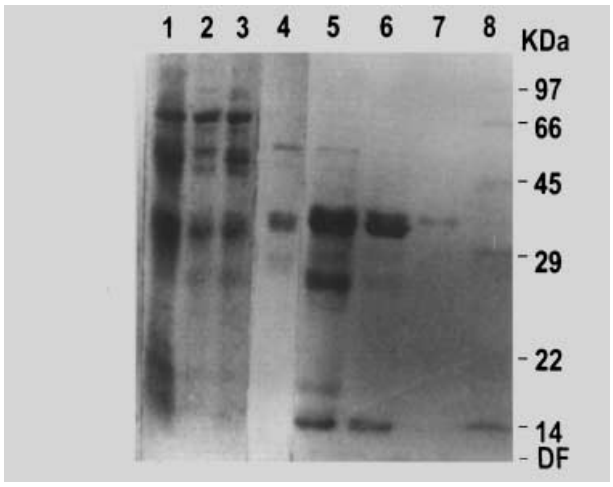

Figure 5. SDS-PAGE (12.5%) of rabbit lung PLTP. Lane 1, cytosol; lane 2, fraction M; lane 3, fraction M of pH 7.4; lanes 4 and 5, Sephadex G-75-eluted fractions (P_3) with maximum PL transfer activity and DEAE-cellulose eluted fractions (Q_4); lane 6, Mono-Q eluted fraction (T_2); lane 7, Protein Pak 200 SW-eluted fraction (R_2/$PLTP_R$); lane 8, molecular-weight protein markers [BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and lysozyme (14.3 kDa)].

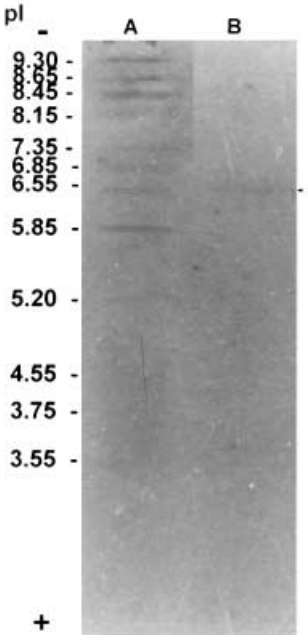

Figure 6. Analytical isoelectrofocussing of $PLTP_R$. Lane A, pI markers (amyloglucosidase pI 3.5, methyl red pI 3.75, soybean trypsin inhibitor pI 4.55, β-lactoglobin A pI 5.2, bovine carbonic anhydrase B pI 5.85, human carbonic anhydrase B pI 6.55, acidic horse myoglobin pI 6.85, basic horse myoglobin pI 7.35, acidic lentil lectin pI 8.15, middle lectin pI 8.45 and basic lectin pI 8.65, trypsinogen pI 9.30).

in table 3. The transfer activity of $PLTP_R$ was found to be 75.63 ± 5.16% in case of ULVs made of PC alone. A statistically insignificant decrease was observed when a small amount of cholesterol was present along with PC in the ULVs. However, in ULVs consisting of PC and cholesterol (at a 50% molar ratio), transfer activity decreased significantly. Furthermore the presence of PI (negatively charged) with PC or PS in ULVs did not alter the transfer activity of $PLTP_R$ significantly.

The lipid composition of the purified surfactant preparation from rabbit lung (8–10 g) lavage showed that the PL content was more than 74% of the total lipid, neutral lipid and cholesterol contents were only 9% each. Effects of different fractions [cytosolic (C), P_3, Q_4 and R_2] in the presence and absence of surfactant liposomes on the uptake of DPPC by freshly isolated type II cells from lungs of different groups of animals (adult rabbits and premature, term and 3-day-old pups) are shown in table 4.

Table 1. Purification of $PLTP_R$ from adult rabbit lung.

Fraction	Volume	Total protein	Yield	Specific activity	Purification	Recovery
	(ml)	(mg)	(%)	(% transfered/h/ per mg protein)	(fold)	(%)
Cytosol	105	957.6	100	29.5	1	100
Fraction M	97	824.6	86	26.3	–	77
Fraction M (pH 7.4)	95	788.5	82	28.3	–	75
$(NH_4)_2SO_4$ fraction (30–70%)	10	326.8	34	33.5	1.1	39
Eluate of Sephadex G-75 column	3	84.3	33.5	127.4	4	37.9
DEAE-cellulose Column-eluted material	2	3.6	0.004	2711.4	92	34.2
Eluate of Mono Q column	1	0.262	0.0003	14984.1	508	13.1
Protein Pak 200 SW column-eluted material	1	0.057	0.0006	24393.5	827	4.3

Table 2. Comparison of the transfer of PC and PI from ULVs to mitochondria in the presence of purified $PLTP_R$.

Radioactively labelled phospholipids in ULVs	Phospholipid transfer activity (%)
Phosphatidylcholine	77.9 ± 3.8
Phosphatidylinositol	9.36 ± 1.22

Mean ± SD of eight to ten observations.

Table 3. Effect of donor membrane composition on PL transfer activity.

Composition of donor vesicles (% molar ratio)	Phospholipid transfer activity (% h)
PC (100)	75.63 ± 5.16
PC : cholesterol (90 : 10)	68.18 ± 2.51*
PC : cholesterol (50 : 50)	54.67 ± 4.68**
PC : PI (50 : 50)	77.95 ± 6.84
PC : PS (50 : 50)	76.8 ± 6.18

Mean ± SD of six to eight observations. * $p < 0.05$, *** $p < 0.001$ (compared to PC liposomes).

An appreciable amount of DPPC was taken up by type II cells in the presence of $PLTP_R$ compared to that of $C/P_3/Q_4$ fractions. Incubation of cells with each fraction, i.e. C, P_3, Q_4 and R_2 along with surfactant liposome revealed significant enhancement in DPPC uptake. The effect of alteration of donor membrane composition on DPPC transfer to alveolar type II cells in the presence of $PLTP_R$ is shown in table 5. In the presence of PC liposomes alone as a donor membrane, the transfer of DPPC to type II cells was greatest in adult rabbits and lowest in premature pups. The uptake of DPPC declined significantly upon incubation of the cells with liposomes containing a high level of cholesterol in all cases except adult rabbits. However, the presence of negatively charged PI in the liposomes significantly enhanced the uptake in 3-day-old pups and adult rabbits but no significant alteration was observed with liposomes containing PS.

Table 4. Effect of different fractions with PL transfer activity on DPPC uptake by type II cells isolated from lungs of different groups of rabbits.

Fraction added	DPPC uptake/10^6 type II cells (nmoles)			
	premature	term pups	3- day-old pups	adult rabbits
C	0.178 ± 0.074	0.482 ± 0.085	0.548 ± 0.120	0.798 ± 0.194
C ± SF	0.183 ± 0.051	0.603 ± 0.069	0.505 ± 0.087	0.909 ± 0.076
P_3	0.241 ± 0.057	0.595 ± 0.112	0.786 ± 0.206	0.968 ± 0.219
P_3 ± SF	0.318 ± 0.037	0.878 ± 0.154	1.021 ± 0.242[a]	1.352 ± 0.258
Q_4	0.425 ± 0.076*	1.209 ± 0.107*	1.389 ± 0.098*	1.891 ± 0.334
Q_4 + SF	0.643 ± 0.081[+,a]	1.473 + 0.052[+,a]	1.625 ± 0.230[a]	2.603 ± 0.362[++,a]
R_2	0.877 ± 0.077***	1.722 ± 0.118**	2.897 ± 0.245***	3.492 ± 0.223***
R_2 + SF	0.174 ± 0.131[++,b]	2.104 ± 0.124[++,b]	3.632 + 0.410[++,b]	4.008 + 0.482[+++,b]

Mean ± S.D. of six to ten observations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ [compared to cytosolic (c) fraction]. [+] $p < 0.05$, [++] $p < 0.01$, [+++] $p < 0.001$ (compared to respective protein fraction). [a] $p < 0.05$, [b] $p < 0.001$ (compared to C + surfactant (SF) liposomes).

Table 5. Effect of donor membrane composition on DPPC transfer to alveolar type II cells isolated from lungs of different sets of animals, in the presence of $PLTP_R$.

Membrane	DPPC uptake/10^6 type II cells (nmoles)			
	premature	term pups	3-day-old pups	adult rabbits
PC	1.013 ± 0.119	1.777 ± 0.202	3.090 ± 0.311	3.780 ± 0.276
PC : cholesterol (50 : 50)	0.624 ± 0.101*	1.120 ± 0.071*	2.213 ± 0.454*	2.564 ± 0.288
PC : PI (50 : 50)	1.401 ± 0.082	2.067 ± 0.328	3.689 ± 0.356**	5.445 ± 0.399*
PC : PS (50 : 50)	1.181 ± 0.092	1.858 ± 0.107	3.154 ± 0.469	3.385 ± 0.471

Mean ± S.D. of six to eight observations. When compared to PC liposomes, * $p < 0.05$, ** $p < 0.01$.

Discussion

Neonatal RDS is known to be associated with abnormal lung surface tension properties. A great deal of work is being carried out to understand the pathophysiology and to prevent this disease [26]. One of the most effective treatments is the use of exogenous surfactant.

Synthesis, storage and secretion of surfactant proceeds solely in alveolar type II cells [27]. These cells also constitute a major route of clearance and reutilization of various surfactant components. Alveolar type II cells have also been reported to contain PLTP, involved in the in vitro transfer of PLs between biological membranes [28]. However, the possible role of such proteins in lung surfactant synthesis, assembly, secretion and reutilization from the alveolar subphase has not been fully explored.

At least four distinct PLTPs with different substrate specificities have been identified in the lung. Among these, one has been reported to be specific for PC and the second is known to transfer both PI and PC. The third was described as non-specific, being able to transfer PI, PC, PS, phosphatidyl ethanolamine (PE) and also cholesterol [29] while the fourth transfer protein was found to be specific for phosphatidyl glycerol [21]. Our purified $PLTP_R$ has been found to transfer PI poorly though it had high PC transfer activity. The initial isolation procedure followed in this study was similar to that reported by Tsao et al. [11], however, a different chromatography combination was chosen to purify $PLTP_R$. The in vivo functions of $PLTP_R$ remain largely unknown. A combination of transfer properties may be involved in PL-sorting mechanism, resulting in a high content of DPPC in lamellar bodies of the alveolar type II cells. Furthermore these proteins have also been suggested to play an important role in membrane translocation of lipids [30].

Earlier observations [20, 21] revealed that DPPC was preferentially taken up by lamellar bodies, following incubation of lung with DPPC-labelled liposomes. Furthermore the rate of PL transfer by lung PLTP was suggested to depend on the physical properties of the membrane interfaces with which the protein interacts and also on the specificity of PLTP. In the present study, incorporation of even small amounts of cholesterol were able to inhibit the transfer activity of the protein, which might be due to an increase in membrane fluidity by the incorporation of cholesterol which in turn could lead to reduced interaction among lipids and protein [31].

Natural membranes contain acidic PLs which have long been recognized to affect the activity of PLTP [32]. PCTP isolated from bovine liver was either stimulated or inhibited by the presence of acidic PLs in liposomes [33]. In contrast, in this study, the presence of acidic PLs such as PI and PS did not significantly affect the transfer activity of the protein. The PITP/PCTP isolated from bovine brain [34], bovine heart [35] and rat liver [36] were found to exist in two subforms with the same molecular weight but of different pI values, which could catalyze the transfer of both PC and PI [34, 36]. Van Paridon et al. [37] have demonstrated the presence of separate binding sites on the proteins for the acyl groups at the Sn-1 and Sn-2 positions of the PLs; however, the Sn-2 acyl chains of PC and PI could apparently share a common binding site on the protein molecule. The mode of action of $PLTP_R$ isolated in the present study may follow the above speculated mechanism.

The present study investigated fetal rabbits at three different developmental windows, i.e. pre-term, term and post-term. An advantage of using this animal model includes the development of various organ systems relative to birth, which has been reported to parallel events in human infants. The present investigation was therefore undertaken with the aim of understanding the role of PLTP as well as the combined use of PLTP and surfactant on the uptake of DPPC by alveolar type II cells isolated at different stages of rabbit lung development.

Several studies have shown that surfactant lipids are taken up by the type II cells and cleared from the alveoli [10]. This route of clearance accounts for > 65% of DPPC uptake and the process contributes to the recycling and reutilization of surfactant PLs. A definite role for the hydrophobic proteins as well as SP-A in enhancing the lipid uptake process has also been proposed [38]. However, the mechanism of and the additional factors that may be involved with, these processes are not clearly understood. The presence of PLTP in the alveolar subphase has also been suggested to assist the reuptake of surfactant lipid components [39]. Kuroki et al. [40] have shown that though the PL to protein ratio of rat type II plasma membrane is slightly higher as compared to that of the hepatocytes, the uptake of liposomal DPPC by the former was two fold greater than the latter. Indirect evidence for such PLTP modification of the membrane has come from studies with mitochondrial membrane [30], platelet plasma membrane and intestinal brush border membrane [41].

In the present study, alveolar type II cells were effectively used as an acceptor membrane and the surfactant liposomal uptake of DPPC by alveolar type II cells was found to be stimulated through the four developmental stages in rabbits in the presence of either purified $PLTP_R$ alone or both $PLTP_R$ and surfactant liposomes. The maximum effect was observed in adult type II cells. These results are comparable to those reported by Kresch et al. [42] using cell cultures of undifferentiated, differentiated and neonatal type II cells, and also fetal rabbit lung [43], where the presence of SP-A enhanced the uptake and reuptake processes of DPPC. The protein purified in the present study was also found to transfer a small amount of PI, which may be responsible for altering the membrane composition in a particular area of type II cells which could subsequently incorporate DPPC with or

without the assistance of $PLTP_R$. Another point to consider is that the type II cells isolated at the development stages used here (26th day of gestation or even at term) have not achieved maturity and hence their membrane compositions are susceptible to greater modulations.

The specific PLTP and the composition of the donor membrane have been reported to determine the modification attainable in the acceptor membrane. Under different experimental conditions (using various lipid compositions as well as *Escherichia coli* lipids), the results obtained by Runquist et al. [44] suggested that the transfer of PC by PCTP was enhanced when insertion of protein-bound PC occurred concurrently with the extraction of a molecule of membrane PC, i.e. in a synchronized one-step catalytic mechanism of PL exchange. Either of the above-mentioned mechanisms might also exist in the type II cells in which PLTP could greatly influence DPPC uptake. Earlier studies on the ontogeny of surfactant synthesis have reported an increase in the PL content in type II cells with advancing developmental stages [45]. In addition, recent experiments [46] have also shown an increase in the PC-synthesizing enzymes along developmental lines. Furthermore, the enzymes regulating PC biosynthesis were shown to be influenced by the presence of PLs in the controlled environment [47]. These studies have also revealed that the PITP/PCTP mediating the intracellular PL transfer was a critical component of this PC-biosynthesizing pathway.

All these factors collectively emphasize that PL biosynthesis and $PLTP_R$ are developmentally regulated. The addition of $PLTP_R$ at any stage could positively modulate the uptake of PC by the alveolar type II cells. Supporting evidence for this contention comes from the fact that treatment of premature babies with surfactant did not show a negative impact on endogenous surfactant synthesis. Furthermore, other studies reports have also ruled out the possibility that exogenous surfactant therapy might inhibit endogenous surfactant synthesis [48] and its secretion in vivo [49] thereby indirectly supporting the results obtained in the present study. Thus, a natural surfactant composition along with a $PLTP_R$ may have biological effects on the type II cells that may prove to be potentially beneficial for the uptake and reutilization of surfactant material as well as for the synthesis of endogenous surfactant PLs.

Acknowledgements. The authors thank the Council of Scientific and Industrial Research (CSIR) for financial assistance.

1 Chida S., Phelps D., Soll R. and Taeusch W. (1991) Surfactant proteins and antisurfactant antibodies in sera from infants with respiratory distress syndrome after surfactant treatment. Pediatrics **88:** 84–89
2 Jacobs H., Jobe A., Agami M. and Conaway D. (1983) The significance of reutilization of surfactant phosphatidyl choline. J. Biol. Chem. **258:** 4159–4165
3 Post M., Batenburg J. J., Schuurmans E. A. J. M. and Van Golde L.M.G. (1980) Phospholipid transfer activity in type II cells isolated from adult rat lung. Biochim. Biophys. Acta **620:** 317–321
4 Tsao F. H. C. (1980) Specific transfer of dipalmitoyl phosphatidyl choline in rabbit lung. Biochem. Biophys. Acta **601:** 415–426
5 Nijssen J. G., Promes L. W., Hardeman D. and Van den Bosch H. (1987) Phospholipid-protein interactions in rat lung lamellar bodies. Biochem. Biophys. Acta **917:** P140–P147.
6 Khan Z. U. and Helmkamp G. M. (1990) Stimulation of choline phospho-transferase activity by phosphatidylcholine transfer protein. J. Biol. Chem. **265:** 700–705.
7 Teerlink T., Van Der Krift J. P., Post M. and Wirtz K.W. A. (1982) Tissue distribution and subcellular localization of phosphatidylcholine transfer protein in rats as determined by radioimmunoassay. Biochem. Biophys. Acta **713:** 61–67.
8 Cunningham E., Thomas G. M. H., Ball A., Hiles I. and Cockcroft S. (1995) Phosphatidylinositol transfer protein dictates the rate of inositol triphosphate production by promoting the synthesis of PIP2. Curr. Biol. **5:** 775-783.
9 Lumb R. H., Bensen B. J. and Clements, J. A. (1988) Transfer of phospholipids by a protein fraction obtained from canine pulmonary lavage. Biochem. Biophy. Acta **963:** 549–552.
10 Moxley M. A., Jacob J. and Longmore W. J. (1991) Uptake and reutilization of surfactant phospholipids by type II cells of isolated perfused lung. Am. J. Physiol. **260:** L269–L273
11 Tsao F. H. C., Tian Q. and Strickland, M. S. (1992) Purification characterization and substrate specificity of rabbit lung phospholipid transfer proteins. Biochem. Biophys. Acta. **1125:** 321–329
12 Dixon M. (1953) A nomogram for ammonium sulfate solutions. Biochem. J. **54:** 457–458
13 Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head bacteriophage T_4. Nature **227:** 680–685
14 Lowry O. H., Rosebrough N. J., Farr A. L. and Randale R. J. (1951) Protein measurement with Folin phenol reagent. J. Biol. Chem. **193:** 265–275
15 Gumbhir K., Sanyal S. N., Minocha R., Wali A. and Majumdar S. (1989) Glucose-6-phosphatase phosphohydrolase activity in guinea pig liver microsomes is influenced by phosphatidyl choline: interactions with cholesterol enriched membranes. Biochem. Biophys. Acta. **981:** 77–84
16 Ikegami M., Jobe A. and Doane G. (1985) Liposomes of dipalmitoyl phosphatidyl choline associated with natural surfactant. Biochim. Biophys. Acta **835:** 352–359
17 Freigns C. S. and Dunn R. T. (1970) A colorimetric method for determination of total serum lipids based on the sulfo-phospho-vanillin reactions. Am. J. Clin. Pathol. **53:** 89–91
18 Marinetti, G. V. (1962) Chromatographic separation, identification and analysis of phosphatides. J. Lipid Res. **3:** 1–11
19 Zak B. (1957) Simple rapid microtechnique of serum total cholesterol. Am. J. Clin. Pathol. **27:** 583–588
20 Read R. J. and Funkhouser J. D. (1983) Properties of a non specific phospholipid transfer protein purified from rat lung. Biochem. Biophys. Acta **752:** 118–126
21 Van Golde L. M. G., Oldenborg V., Post M., Batenburg J. J., Poorthuis B. J. H.M. and Wirtz K. W. A. (1980) Phospholipid transfer proteins in rat lung. J. Biol. Chem. **255:** 6011–6013
22 Hudson L. and Hay F. C (1989) Affinity techniques for molecules and cells. In: Practical Immunology, 3rd edn, pp 315–316, Elaine and Frances (eds), Blackwell, Oxford.
23 Dobbs L.G. (1990) Isolation and culture of alveolar type II cells. Am. J. Physiol. **258:** L134–L147
24 Wright J. R., Wager R. E., Hawgood S., Dobbs L. and Clements J. A. (1987) Surfactant apoprotein Mr = 26,000–36,000 enhances uptake of liposomes by type II cells. J. Biol. Chem. **262:** 2888–2894

25 Ispen J. and Feige l P (1970) Boncrofts Introduction to Biostatistics, 2nd edn, Harper and Row, New York
26 Van Golde L. M. G., Batenburg J. J. and Robertson B. (1988) The pulmonary surfactant system: biochemical aspects and functional significance. Physiol. Rev. **68:** 374–455
27 Pettenazzo A., Ikegami M., Seidner S. and Jobe A. (1988) Clearance of surfactant phosphatidyl choline from adult rabbit lungs. J. Appl. Physiol. **64:** 1210–1227
28 Kresch M. J., Gipriani L. A., Lu H. and Christian C. (1994) Developmental regulation of re-uptake of phosphatidyl choline by type II alveolar epithelium. Biochem. Biophys. Acta. **1210:** 167–173
29 Zilversmit D. B. (1984) Lipid transfer proteins. J. Lipid Res. **25:** 1563–1569
30 Dolis D., Anton I. P. M. and Kruijff B. de (1996) Transmembrane movement of phosphatidyl choline in mitochondrial outer membrane vesicles. J. Biol. Chem. **271:** 11879–11883
31 Somerharju P. J., Van Loonm D. and Wietzm K. W. (1987) Determination of the acyl chain specificity of the bovine liver phosphatidylcholine transfer protein: application of pyrene labelled phosphatidylcholine species. Biochemistry **22:** 7193–7197
32 Lumb R. H. (1989) Phospholipid transfer proteins in mammalian lung. Am. J. Physiology **257:** L190–L194
33 Wirtz K. W. A. and Gadella T. W. J. Jr (1990) Properties and modes of action of specific and non specific phospholipid transfer proteins. Experentia **46:** 592
34 Helmpkamp G. M. (1986) Phospholipid transfer proteins: mechanisms of action. J. Bioenergy Biomemb. **18:** 71–91
35 Dicorleto P. E., Warach J. B. and Zilversmit D. B. (1979) Purification and characterization of two phospholipid exchange proteins from bovine heart. J. Biol. Chem. **254:** 7795–7802
36 Wirtz K. W. A. (1982) Phospholipid transfer proteins. Klin. Wochenschr. **69:** 105–111
37 Van Paridon P. A., Gadella T. W. J., Somerhaju P. J. and Wirtz K. W. A. (1987) On the dual relationship between the dual specificity of the bovine brain phosphatidylinositol transfer and membrane phosphatidylinositol levels. Biochem. Biophys. Acta **903:** 68–77
38 Horowitz A. D., Moussavian B. and Whitsett J. A. (1966) Roles of SP-A, SP-B and SP-C in modulation of lipid uptake by pulmonary epithelial cells in vitro. Am. J. Physiol. **270:** L69–L79
39 Koumanov K., Boyanov A., Neitcheva T. and Markovska T. (1981) Purification et specificite de la protein exchange use de phospholipides du surfactant alveolaire du rat. Bull. Eur. Physiopathol. Respir. 17: 411–418
40 Kuroki Y., Shiratori M., Ogassawaia Y., Hattori A., Tsunezawa W., Honma T. et al. (1996) Interaction of phospholipid liposomes with plasma membrane isolated from alveolar type II cells: effect of pulmonary surfactant protein A. Biochem. Biophys. Acta **1281:** 45–52
41 Brasituss T. A., Dhaiya R., Dudeja P. K. and Bissonnette B. M. (1988) Cholesterol modulates alkaline phosphatase activity of rat intestinal microvillus membranes. J. Biol. Chem. **263:** 8592–8597
42 Kresch M. J., Dynia D. W. and Gross I. (1987) Culture of differentiated and undifferentiated type II cells from fetal rat lung. Biochim. Biophys. Acta **930:** 19–32
43 Scott J. E. (1992) Phosphatidyl choline synthesis, secretion, and reutilization during differentiation of the surfactant producing type II cells from fetal rabbit lungs. Exp. Lung. Res. **18:** 563–580
44 Runquist E. A. and Helmkamp G. M. Jr (1988) Effect of acceptor membrane phosphatidyl choline on the catalytic activity of bovine liver phosphatidyl choline transfer protein. Biochem. Biophys. Acta. **940:** 21–32
45 Griese M., Gobran L. I. and Rooney S. A. (1992) Ontogeny of surfactant secretion in type II pneumocytes of fetal newborn and adult rats. Am. J. Physiol. **262:** L337–L343
46 Zimmerman L. J. I., Lee W. S. and Post M. (1995) Regulation of CTP: phosphocholine cytidyl transferase by cytosolic lipids in rat type II pneumocytes during development. Pediatr. Res. **38:** 864–869
47 Kent C., Carman G. M., Spince M. W. and Douehan W. (1991) Regulation of eukaryotic phospholipid metabolism. FASEB J. **5:** 2258–2266
48 Ikegami M., Jobe A., Yamada T., Priestly A., Ruffine L., Rider E. et al. (1989) Surfactant metabolism in surfactant treated preterm ventilated lambs. J. Appl. Physiol. **67:** 429–437
49 Hallman M., Merritt T. A., Pohjavuori M. and Gluck L. (1986) Effect of surfactant substitution on lung effluent phospholipid in respiratory distress syndrome: evaluation of surfactant phospholipid turnover, pool size and the relationship to severity of respiratory failure. Pediatr. Res. **20:** 1228–1235