Phospholipid-transfer proteins and their mRNAs in developing rat lung and in alveolar type-II cells

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Gene expression of non-specific lipid-transfer protein (nsL-TP; identical with sterol carrier protein 2) and phosphatidylinositol-transfer protein (PI-TP) was investigated in developing rat lung. During the late prenatal period (between days 17 and 22) there is a 7-fold increase in the level of nsL-TP and a 2-fold rise in that of PI-TP. The prenatal increases in the levels of nsL-TP and PI-TP are accompanied by parallel increases in the levels of their mRNAs, indicating pretranslational regulation. Compared with whole lung, isolated alveolar type-II cells are enriched in nsL-TP and its mRNA, but not in PI-TP and its mRNA. The observation that the levels of nsL-TP and its mRNA in rat lung show a pronounced increase in the period of accelerated surfactant formation, together with the observation that the surfactant-producing type-II cells are enriched in nsL-TP and its mRNA, suggest that nsL-TP plays a role in the metabolism of pulmonary surfactant.

INTRODUCTION

Pulmonary surfactant is synthesized and secreted by the alveolar type-II epithelial cells [1,2]. This material, which is required to lower the surface tension at the alveolar surface in order to prevent alveolar collapse, consists of 90% lipids and 10% proteins [1–3]. The major part of the surfactant lipids consists of phospholipids, of which phosphatidylcholine (PC) makes up 70–85% and, in most mammalian species studied, phosphatidylglycerol (PG) constitutes 5–10% [2]. The largest amount of neutral lipid is made up of cholesterol [2]. The production of surfactant is greatly accelerated towards the end of gestation [4]. Before their secretion from type-II cells, the surfactant lipids are stored in the lamellar bodies [5,6]. From autoradiographic studies [6] it is known that PC is transferred to these storage organelles after its synthesis at the endoplasmic reticulum. The details of the intracellular transport of surfactant lipids from their site of synthesis to the lamellar bodies are still poorly understood (for review, see refs. [1,2]). The observations that there is a peak in PC-transfer activity in fetal mouse [7] and rat [8] lung in the period of accelerated surfactant formation led to the suggestion that phospholipid-transfer proteins may be involved. A second indication that phospholipid-transfer proteins have a role in the surfactant system was the observation of a significant correlation between the amount of PC-transfer activity in six vertebrate species and both the alveolar surface area and the amount of surface-active material [9]. A third indication came from the observations that the PC- and PG-transfer activities are much higher in type-II cells than in the average lung cell [10,11].

Phospholipid-transfer proteins, which *in vitro* facilitate the transfer of phospholipids between biological membranes, are found in many cell types. The three best-studied types are: (1) PC-transfer protein (PC-TP), which transfers only PC; (2) PI-transfer protein (PI-TP), which has a preference for PI, but can also carry PC; and (3) non-specific lipid-transfer protein (nsL-TP) [or sterol carrier protein 2 (SCP_2)], which transfers phospholipids, glycolipids and cholesterol [12,13]. Using immunological techniques, it was found that all three proteins are present in the lung [8,14,15]. It was shown that the peak in PC-transfer activity in rat lung in the perinatal period coincides with a peak in the amount of PC-TP as determined by radioimmunoassay [8]. Given the ability of PI-TP and nsL-TP to transfer PC as well, one cannot exclude a contribution of these phospholipid-transfer proteins to the total PC-transfer activity in the lung. The present study was undertaken to investigate whether nsL-TP and PI-TP also increase in rat lung in the perinatal period and, if so, whether this is brought about at a pretranslational level. Moreover, the levels of nsL-TP and PI-TP and their mRNAs in isolated type-II cells were compared with those in whole lung.

MATERIALS AND METHODS

Materials

The following plasmids were used to make probes for Northern-blot hybridization analysis: a clone containing a 724 bp insert encoding rat liver nsL-TP [16], clone PI-12 (1.7 kb insert encoding rat brain PI-TP [17]), clone pR ME1 {2.4 kb insert encoding rat liver malic enzyme (ME) [18]} and clone pHcGAP {1.2 kb insert encoding human liver glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [19]}. [α-^{32}P]dATP (3000 Ci/mmol) and ^{125}I-labelled goat anti-(rabbit IgG) (7 μCi/μg) were obtained from New England Nuclear (Wilmington, DE, U.S.A.). Goat anti-(rabbit IgG) antibody conjugated to alkaline phosphatase, 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (BCIP), *p*-Nitro Blue Tetrazolium chloride (NBT) and molecular-mass standards for SDS/PAGE were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Ficoll (400 kDa) and dextran sulphate (~ 500 kDa) came from Pharmacia (Uppsala,

Abbreviations used: nsL-TP, non-specific lipid-transfer protein; PI-TP, phosphatidylinositol-transfer protein; PC, phosphatidylcholine; PG, phosphatidylglycerol; BCIP, 5-bromo-4-chloro-3-indolyl phosphate (toluidine salt); NBT, *p*-Nitro Blue Tetrazolium chloride; ANOVA, analysis of variance; SNK, Student–Newman–Keuls; SCP_2, sterol carrier protein 2; 1 × SSPE, 0.15 M NaCl + 1 mM EDTA + 10 mm NaH_2PO_4 (pH 7.4); ME, malic enzyme; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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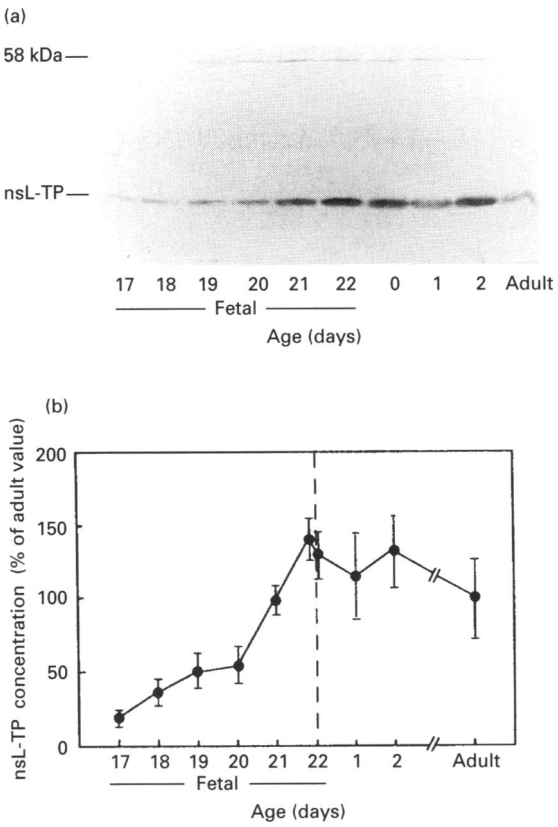

Figure 1 Relative levels of nsL-TP in rat lung during development

(**a**) Samples (40 μg of protein) of whole lung homogenate were analysed as described in the Materials and methods section. The lane marked fetal age 22 days corresponds to animals not yet born on the day of term, whereas the lane marked age 0 corresponds to animals born spontaneously and processed on that day. The data are representative of results obtained in four series with preparations of independent litters or adult animals. (**b**) The intensities of the bands on the immunoblots were quantified by densitometry. Term is emphasized by a vertical dashed line at 22 days fetal age. The data points to the left of this line indicate results with animals not yet born on the day of term, whereas the data points to the right of the line indicate results with animals born spontaneously on that day. The values given represent means $\pm$ S.E.M. obtained with four litters or four adults. The mean values for the adult animals were taken as 100%. ANOVA indicated that there were significant differences among the rats at different ages ($F < 0.0001$). According to the SNK test the value at fetal day 21 differs from that at fetal day 17, the values at day 22 (fetal and neonatal) and at neonatal day 2 differ from those at fetal days 17, 18, 19 and 20, the value at neonatal day 1 differs from that at fetal days 17 and 18, and the adult value differs from that at fetal day 17 ($P < 0.05$).

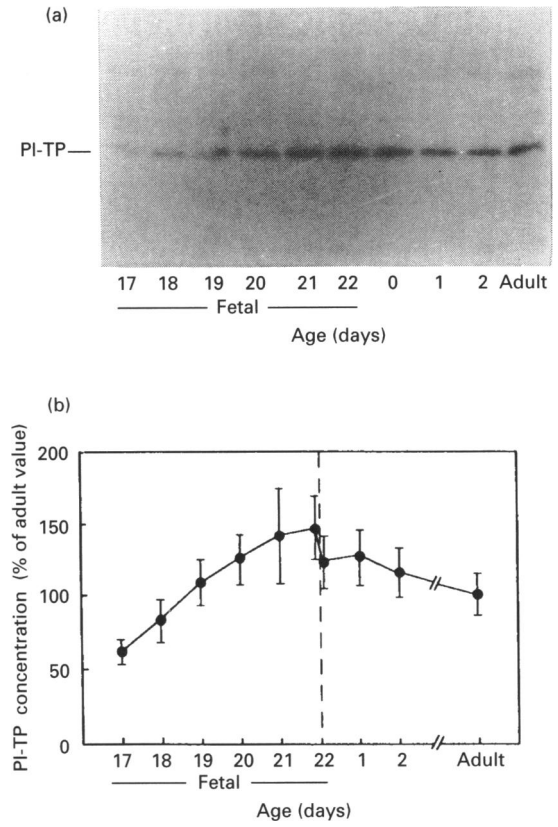

Figure 2 Relative levels of PI-TP in rat lung during development

(**a**) Samples of whole lung homogenates (10 μg of protein) were subjected to SDS/PAGE, immunoblotting and autoradiography as described in the Materials and methods section. The data represent results obtained in four series with preparations of independent litters or adult animals. (**b**) The intensities of the PI-TP bands on the autoradiograms were quantified by laser densitometry. The values given represent means $\pm$ S.E.M. ($n = 4$). The mean value for the adult animals was taken as 100%. ANOVA indicated that there were significant differences among the rats at different ages ($F < 0.0001$). According to the SNK test the values at fetal day 19 and higher ages differ from that at fetal day 17, the values at fetal day 20 through neonatal day 1 differ from those at fetal days 17 and 18, and the adult value differs from those at fetal days 17, 21 and 22 ($P < 0.05$).

Sweden). Nitrocellulose and Nylon (Nytran N) membranes (pore size 0.45 μm) were supplied by Schleicher and Schuell (Dassel, Germany). Pregnant Wistar rats of known gestation time (term is at day 22) and adult male rats (180–200 g) were obtained from the Central Animal Laboratory, Utrecht University.

Tissue collection

In studies on development, timed-pregnant rats were killed by CO_2 asphyxiation and the fetuses delivered by uterine section. Neonates were born spontaneously on day 22 of gestation and remained with their mother until they were killed. On the day of term, both fetuses and neonates were studied. No effort was made to determine exactly how much time had elapsed since the moment of birth of the neonates. Both fetuses and neonates were killed by decapitation. The lungs were immediately removed and freed of large airways. Lung tissue from adult rats was obtained after CO_2 asphyxiation. For the isolation of RNA the lung tissue was immediately frozen in liquid nitrogen and stored at -80 °C. Tissue from each litter was stored together in one vial. Random samples (0.3–0.4 g of tissue) were taken from these vials for RNA isolation.

Isolation of type-II cells

Type-II cells were isolated from adult rats as described by Dobbs et al. [20].

Production of antibodies

Affinity-purified antibodies raised in rabbits against rat liver nsL-TP were prepared as described previously [21]. Protein A-purified antibody against PI-TP was raised as described [22] against synthetic peptides representing the amino acid sequences of predicted epitopes in rat brain PI-TP [17].

Immunoblotting

For immunoblotting experiments, homogenates (20%, w/v) of fresh lung tissue were prepared at 0 °C in buffer containing 0.25 M sucrose, 1 mM EDTA and 10 mM Tris/HCl, pH 7.4 (SET) using a Potter–Elvehjem homogenizer. Part of the homogenate was centrifuged for 10 min at 1000 *g* (4 °C) to remove nuclei and cell debris. From the supernatant a particulate fraction was obtained by centrifugation for 1 h at 100000 *g* (4 °C). After resuspension in SET, this fraction, together with the 100000 *g* supernatant and the remainder of the homogenate, was stored at −20 °C until analysis. Type-II cells were washed twice with PBS and disintegrated in SET by sonication with a Soniprep 150 ultrasonic disintegrator (MSE Scientific Instruments, Crawley, Sussex, U.K.), using the exponential microprobe (3 mm tip diameter; 4 × 15 s at frequency 23 kHz; amplitude 6 μm; 0 °C). The protein content of the various preparations was determined by the method of Lowry et al. [23] with BSA as standard.

For determination of proteins reactive with anti-nsL-TP antibodies, samples were submitted to SDS/PAGE on 15% (w/v) gels [24] in a Mini-Protean II dual-slab cell (Bio-Rad), followed by blotting on to nitrocellulose in a Mini Trans-blot cell (Bio-Rad) at 100 V for 1.5 h in transfer buffer [25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3]. Immunological detection was carried out with goat anti-(rabbit IgG) antibody conjugated to alkaline phosphatase as the second antibody. BCIP and NBT were used as substrates according to the protocol recommended by the supplier. The intensities of the bands obtained were quantified by densitometry, using an Ultroscan XL laser densitometer (LKB, Bromma, Sweden).

Proteins reactive with anti-PI-TP antibodies were determined by SDS/PAGE on 12% (w/v) gels [24], followed by blotting on to nitrocellulose as described above and immunological detection using ^{125}I-labelled goat anti-(rabbit IgG) as the second antibody. Autoradiograms of the blots were made at −80 °C. The intensities of the immunoblot signals on the autoradiograms were quantified by densitometry as above.

Isolation and Northern-blot hybridization of RNA

For the isolation of RNA from type-II cells, the cells (around 3×10^7 per preparation) were washed twice with PBS and collected by centrifugation. They were lysed in 3 ml of homogenization buffer consisting of 4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sodium *N*-laureoylsarcosine, 0.1% antifoam A and 0.1 M 2-mercaptoethanol [25]. The viscous suspension was homogenized with an Ultra-Turrax mixer (Janke and Kunkel, Staufen im Breisgau, Germany) at 0 °C. For the isolation of RNA from lung tissue, 0.3–0.4 g of frozen tissue was homogenized in 3 ml of the same medium. The RNA was separated from the homogenates by CsCl centrifugation [25]. RNA was quantified by measuring A_{260}. Aliquots containing equal amounts of total RNA were resolved by electrophoresis through a 1.2% agarose–formaldehyde gel [26]. After separation, the RNA was transferred to Nylon membrane by capillary blotting. Staining with Methylene Blue showed that equal amounts of RNA were present on the blot in each lane. The blots were heated at 80 °C for 2 h. They were prehybridized at 42 °C for 3 h in buffer containing 6× standard saline phosphate EDTA (SSPE, 1× SSPE is 0.15 M NaCl, 1 mM EDTA, 10 mM NaH_2PO_4, pH 7.4), 1% SDS, 10× Denhardt's solution [1× Denhardt's solution is 0.2 mg each of BSA, Ficoll and poly(vinylpyrrolidone)/ml of water] and 0.1 mg/ml denatured phenol-extracted salmon sperm DNA. Subsequently, they were hybridized at 42 °C for 16–18 h in 50 ml of a medium containing 6× SSPE, 1% SDS, 5× Denhardt's solution, 0.1 mg/ml denatured phenol-extracted salmon sperm DNA, 10% (v/v) dextran sulphate, 50% (w/v) formamide and 0.25 μg of ^{32}P-labelled cDNA probe. The ^{32}P-labelled probes (approx. 4×10^8 c.p.m./μg of DNA) were prepared by random priming [27] from [α-^{32}P]dATP and cDNA inserts that had been excised from the plasmids with the appropriate restriction enzymes. After the hybridization, the blots were washed twice at room temperature for 5 min in 6× SSPE/1% SDS, twice at 42 °C for 20 min in 2× SSPE/0.3% SDS and twice at 45–50 °C for 30 min in 0.2× SSPE/0.2% SDS. Autoradiograms of the blots were made at −80 °C. The intensities of the hybridization signals were quantified by densitometry as above.

Statistical analysis

Statistical analysis was carried out by analysis of variance (ANOVA). In those cases where the *F* test indicated that there was a significant difference ($F < 0.05$) among groups, comparisons between groups were made using the Student–Newman–Keuls (SNK) test.

RESULTS

Relative levels of nsL-TP and PI-TP in whole rat lung during development

Figure 1(a) shows the relative levels of nsL-TP (13 kDa) in homogenates of rat lung at different stages of development. It is clear that the level of this protein shows a developmental increase. The same is seen for a 58 kDa protein that reacts with the anti-[nsL-TP (SCP_2)]antibody. This protein, which is also referred to as SCP_x [28] and is found in many different tissues [14], contains the entire sequence of nsL-TP (including a 2 kDa presequence) at

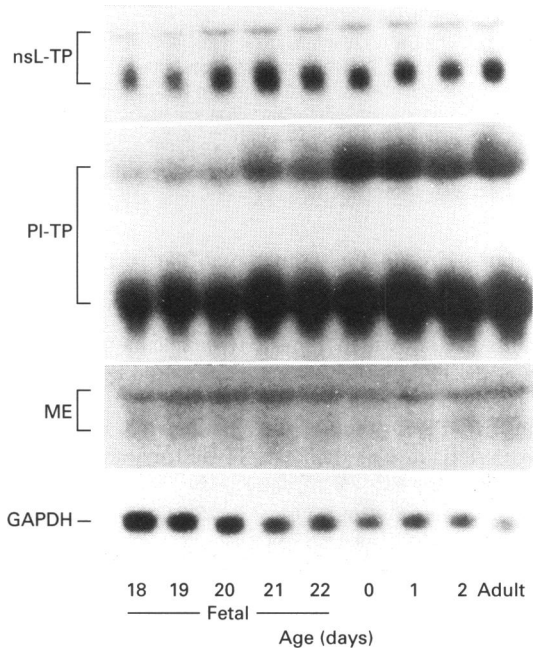

Figure 3 Northern-blot hybridization analysis of the relative levels of mRNAs encoding nsL-TP, PI-TP, ME and GAPDH in rat lung during development

Samples of 50 μg of total RNA were resolved, blotted, hybridized and autoradiographed as described in the Materials and methods section.

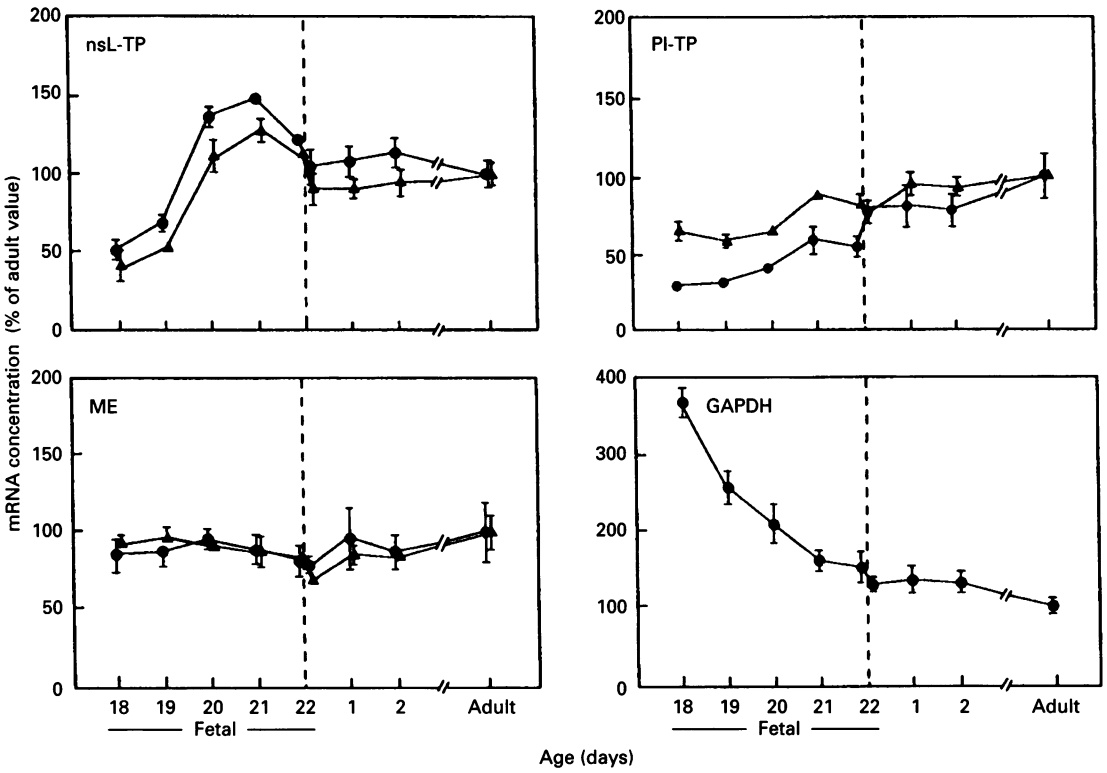

Figure 4 Relative levels of the mRNAs encoding nsL-TP, PI-TP, ME and GAPDH in rat lung during development

Samples of 50 μg of total RNA were subjected to Northern-blot hybridization. The intensities of the hybridization signals on the autoradiograms were quantified by densitometry. In the respective panels the data points for the 1.4 kb (●) and 0.8 kb (▲) mRNAs of nsL-TP, for the 3.5 kb (●) and 1.7 kb (▲) mRNAs of PI-TP and for the 3.4 kb (●) and 2.4 kb (▲) mRNAs of ME are slightly shifted relative to each other for clarity. The values given represent means ± S.E.M. obtained with four litters or four adults. Where no error bar is shown, the S.E.M. was too small to depict. In each panel the mean value found for the adult animals was taken as 100%. There were significant differences among the rats of different ages for the mRNAs of nsL-TP, PI-TP and GAPDH (ANOVA, $F < 0.0001$). For the 1.4 kb nsL-TP mRNA, the values at fetal days 18 and 19 differ significantly from each other and from all other values, the value at fetal day 20 differs from those at fetal days 18 and 19 and post partum, the value at fetal day 21 differs from those at fetal days 18, 19 and 22 and post partum and the value at fetal day 22 differs from those at fetal days 18, 19 and 21 and from the adult value (SNK, $P < 0.05$). For the 0.8 kb nsL-TP mRNA the values at fetal days 18 and 19 differ from those at later days and the value at fetal day 21 differs from those at fetal days 18 and 19 and post partum (SNK, $P < 0.05$). For the 3.5 kb PI-TP mRNA, the values at fetal days 18 and 19 differ from those at fetal day 21 and post partum, the values at fetal days 20 and 22 differ from those at post partum, the value at fetal day 21 differs from those at fetal days 18 and 19 and from the adult value, and the value at neonatal day 1 differs from those at fetal days 18, 19, 20 and 22 and from the adult value (SNK, $P < 0.05$). For the 1.7 kb PI-TP mRNA, the values at fetal days 18 and 20 differ from those at fetal day 21 and neonatal days 1 and 2 and from the adult value, the value at fetal day 19 differs from that at fetal day 21 and higher ages, and the values of day-22 pups (fetal and neonatal) differ from that at fetal day 19 and from the adult value (SNK, $P < 0.05$).

its C-terminus [28–30]. For a more quantitative analysis, the nsL-TP spots on the blots of the homogenates were scanned with a densitometer and the mean value at every age was calculated. The results are shown in Figure 1(b). It can be seen that the amount of nsL-TP in rat lung increases 7-fold between fetal day 17 and term. In the lung, as in other extrahepatic tissues of the adult rat, nsL-TP is predominantly associated with membranes [14]. To investigate whether nsL-TP redistributes between the membrane and the cytosolic fractions during development, these fractions were tested separately. The developmental increase in the level of nsL-TP was observed both in the particulate fraction and in the 100000 *g* supernatant fraction (results not shown). However, in the 100000 *g* supernatant fraction, the signal was much weaker than in the particulate fraction. This indicates that, at all developmental stages, most of the nsL-TP is membrane-associated.

Immunoblot analysis using PI-TP antibody (Figure 2a) shows that the amount of PI-TP in rat lung also increases with development. Quantification by densitometry shows that the increase between fetal day 17 and term is approx. 2-fold (Figure 2b). From a comparison of Figures 1(b) and 2(b) it is clear that the prenatal rise in the level of nsL-TP is distinctly more pronounced than that of PI-TP.

Relative levels of the mRNAs encoding nsL-TP and PI-TP in whole rat lung during development

On Northern-blot hybridization analysis of total rat liver RNA with an nsL-TP cDNA probe, Seedorf and Assmann [28] observed 0.8, 1.4, 2.1 and 2.7 kb mRNA bands. These authors concluded that the 2.1 and 2.7 kb mRNAs encode the 58 kDa protein SPC_x, that the 0.8 and 1.4 kb mRNAs encode the 15 kDa nsL-TP precursor protein which is cleaved to yield nsL-TP itself, and that the 1.4 and 2.7 kb mRNA species are generated by alternative polyadenylation of the 0.8 and 2.1 kb mRNAs respectively. We observed the same bands when analysing total or polyadenylated RNA from rat liver (results not shown) and lung. However, whereas the lung preparations yielded 0.8 and 1.4 kb bands (Figure 3) that were only slightly less intense than those found on analysis of liver preparations, bands corresponding to the larger RNAs seen in lung preparations were very faint compared with those seen in liver preparations. In our

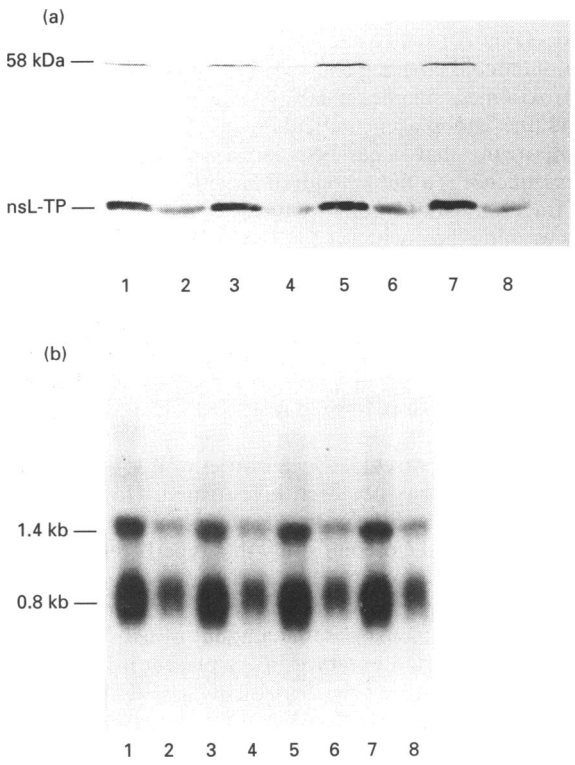

Figure 5 Relative levels of nsL-TP and the related 58 kDa protein and of nsL-TP mRNA in isolated type-II cells and whole lung

(**a**) Immunoblot analysis of nsL-TP and the related 58 kDa protein. Samples (40 μg of protein) of four independent type-II cell preparations and of four independent whole-lung homogenates were analysed with anti-(rat liver nsL-TP) antibody as described in the Materials and methods section. Lanes 1, 3, 5 and 7, type-II cells; lanes 2, 4, 6 and 8, whole lung. (**b**) Northern-blot hybridization analysis of the relative levels of nsL-TP mRNA in isolated type-II cells and whole lung. Samples of 25 μg of total RNA from four independent type-II cell preparations and four separate pairs of rat lungs were analysed as described in the Materials and methods section. Lanes 1, 3, 5 and 7, type-II cells; lanes 2, 4, 6 and 8, whole lung.

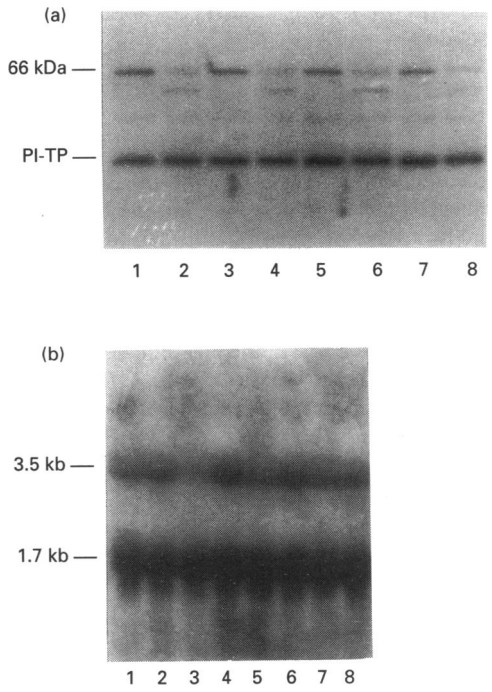

Figure 6 Relative levels of PI-TP and its mRNA in isolated type-II cells and whole lung

(**a**) Immunoblot analysis of PI-TP. Samples (10 μg of protein) of four independent type-II cell preparations and of four independent whole-lung homogenates were analysed with anti-PI-TP antibody as described in the Materials and methods section. Lanes 1, 3, 5 and 7, type-II cells; lanes 2, 4, 6 and 8, whole lung. (**b**) Northern-blot hybridization analysis of PI-TP mRNA. Samples of 25 μg of total RNA from four independent type-II cell preparations and four separate pairs of rat lungs were analysed as described in the Materials and methods section. Lanes 1, 3, 5 and 7, type-II cells; lanes 2, 4, 6 and 8, whole lung.

Northern-blot hybridization analyses of lung RNA with the PI-TP cDNA probe, we observed bands of approx. 1.7 and 3.5 kb (Figure 3), which agrees with the observation of 1.9 and 3.4 kb bands by Dickeson et al. [17] on analysis of total RNA from various rat tissues.

The relative levels of the mRNAs encoding nsL-TP and PI-TP during development are shown in Figure 3. ME mRNAs (2.4 and 3.4 kb) and GAPDH mRNA (1.4 kb) were used as internal controls. It can be seen that nsL-TP mRNA and PI-TP mRNA show a developmental increase, whereas such an increase is not seen for the mRNAs of ME and GAPDH. In autoradiograms exposed for a much longer time than required for visualization of the nsL-TP signals, it could be seen that the abundance of the mRNAs encoding SCP_x also rose between fetal day 18 and term (not shown). For a more quantitative analysis of the changes in the levels of the mRNAs encoding nsL-TP, PI-TP, ME and GAPDH, the spots in the autoradiogram were scanned with a densitometer and the mean value at every age was calculated. The results of these measurements are combined in Figure 4. The analysis shows that nsL-TP mRNAs increase approx. 3-fold between fetal days 18 and 21 and subsequently decrease slightly. PI-TP mRNAs also increase significantly in the perinatal period, but this increase is less pronounced than that for the nsL-TP mRNAs. The abundance of GAPDH mRNA decreases significantly in the prenatal period, whereas, as has been observed previously [31], ME mRNAs show no change with development. This indicates that the increases observed for the abundance of nsL-TP and PI-TP mRNA do not simply reflect non-specific changes occurring in the level of mRNAs in general. The observations that nsL-TP and PI-TP mRNAs increase with development in the prenatal rat lung indicate that the increases seen for nsL-TP and PI-TP at the protein level (Figures 1 and 2) are, at least partially, brought about by pretranslational regulation.

Relative levels of nsL-TP and PI-TP and their mRNAs in isolated type-II cells and whole lung

Immunoblot analysis with anti-nsL-TP antibody shows that, compared with whole lung, type-II cells are enriched in nsL-TP and the related 58 kDa protein (Figure 5a). Northern-blot hybridization experiments demonstrate that the type-II cells are also enriched in nsL-TP mRNA (Figure 5b). Figure 6(a) shows, by immunoblot analysis using anti-PI-TP antibody, that type-II cells are not enriched in PI-TP compared with whole lung. The antibody also reacts with a protein of approx. 66 kDa which has a relatively high abundance in type-II cells. In whole lung this protein has a much lower abundance (Figure 6a) and is sometimes not even detected (Figure 2). The relationship of this 66 kDa protein to PI-TP is unknown. From Figure 6(b) it can be seen

that, compared with whole lung, type-II cells are not enriched in PI-TP mRNA.

DISCUSSION

From the preceding section it is clear that, during prenatal rat lung development, there is a rise in the amount of nsL-TP (Figure 1b) and its mRNA (Figure 4) in the period of accelerated surfactant production. These observations, together with the observation that, compared with whole lung, type-II cells are enriched in nsL-TP (Figure 5a) and its mRNA (Figure 5b), suggest that nsL-TP plays a role in the metabolism of pulmonary surfactant.

nsL-TP was originally purified as a cytosolic factor that stimulates the transfer of phospholipids between membranes. The protein is identical with SCP_2, which was purified as a non-enzymic stimulator of cholesterol biosynthesis. The stimulatory effect in this case is due to the transfer of the lipid intermediate to the site of conversion, i.e. delivering the substrate to the membrane-bound enzymes (for recent reviews, see refs. [13,32,33]). However, as observations from experiments *in vitro* cannot be extrapolated directly to the situation *in vivo*, the physiological function of the protein is still unclear. Nevertheless, nsL-TP has been found in all mammalian tissues tested so far and the amino acid sequence of the protein is highly conserved among species (more than 90% identity), which suggests an important task for the protein in the cell. Recent studies on the regulation of nsL-TP gene expression support the concept [13,32] that nsL-TP plays a physiological role in cholesterol metabolism. It was observed that, during prenatal development of rat liver and small intestine, the abundance of the 0.8 kb nsL-TP mRNA has a peak at fetal day 19 [34]. This developmental pattern in liver and intestine is similar to that of the mRNAs encoding hydroxymethylglutaryl-CoA synthase, prenyl transferase and hydroxymethylglutaryl-CoA reductase [35], suggesting that, like these enzymes, nsL-TP is required for cholesterol synthesis. After partial hepatectomy or unilateral nephrectomy, levels of nsL-TP in the remaining liver tissue or contralateral kidney respectively were found to increase 5-fold, suggesting involvement of the protein in compensatory cell growth, a process requiring cholesterol synthesis for membrane formation [34]. A role for nsL-TP in cholesterol synthesis or cholesterol utilization for steroidogenesis is supported by the recent observations [36] that, in the rat ovary, nsL-TP mRNA is primarily localized in the cells with highest steroidogenic activity, and the treatment of immature rats with gonadotropin leads to an increased level of nsL-TP mRNA in the ovaries.

In a study by Van Heusden et al. [14], the amount of nsL-TP/mg of protein was found to be highest in liver, lung and adrenals. The high level in lung was not confirmed by a recent study [34], although a high level of nsL-TP mRNA was observed. We have no explanation for this discrepancy except to assume that the authors failed to effectively homogenize the lung tissue. It was found that, on subcellular fractionation of various rat tissues, in all tissues except liver the bulk of nsL-TP was localized in the membrane fractions [14]. In liver, adrenal gland and testes, this membrane fraction was identified as peroxisomes by immunogold labelling using a specific antibody raised against nsL-TP [37–40]. A peroxisomal localization of nsL-TP is in good agreement with the presence of the C-terminal peroxisomal targeting signal Ala-Lys-Leu [28–30]. nsL-TP (13 kDa) is synthesized as a 15 kDa precursor [41,42], and processing of this precursor depends on the presence of functional peroxisomes [43]. In the absence of peroxisomes, the precursor is rapidly degraded [43]. In spite of these observations, the distribution of nsL-TP in the cell is still open to debate for several reasons. (1) Immunogold labelling showed, in addition to the labelling in the peroxisomes, significant labelling in mitochondria, the cytosol and the endoplasmic reticulum [37–39]. In this respect, it is noteworthy that it has been suggested that the 20-amino acid presequence is a mitochondrial targeting signal [37]. (2) In testis L-fraction, nsL-TP could only be released from the membranes by washing them with 0.1 M Na_2CO_3 (pH 11.5), suggesting that, in this tissue at least, nsL-TP is a peripheral membrane protein [14]. Furthermore, membrane-bound nsL-TP in testis and Chinese hamster ovary cells was sensitive to trypsin treatment, suggesting exposure to the cytosol [14,44]. (3) In Sertoli cells, which probably do not contain peroxisomes, the protein was present in its mature form in relatively high amount, throughout the cell [39].

So far the intracellular localization of nsL-TP in lung type-II epithelial cells has not been investigated. However, it is likely that at least part of the protein is present in the peroxisomes. The observation in the present study that type-II cells are enriched in nsL-TP, together with earlier observations that type-II cells are relatively rich in peroxisomes [45,46], is in line with this assumption. The increase in the amount of nsL-TP in the lung in the prenatal period observed in the present investigations could therefore be related to the increase in the number of peroxisomes in type-II cells shortly before birth [47]. If nsL-TP is a peroxisomal protein in type-II cells, the initial assumption that nsL-TP may be involved in the transfer of PC from the endoplasmic reticulum to the lamellar bodies (see the Introduction) seems unlikely. However, it is possible that, in type-II cells, similarly to the probable case in liver and intestine (see above), nsL-TP is involved in the production of cholesterol. Cholesterol is the most abundant neutral lipid in surfactant. In the present investigations, the abundance of nsL-TP mRNA in the developing rat lung was found to show a peak just before term. This is very similar to the developmental pattern in rat lung of the mRNAs encoding hydroxymethylglutaryl-CoA synthase and hydroxymethylglutaryl-CoA reductase, two enzymes involved in cholesterol synthesis [35]. Although it was until recently thought that cholesterol synthesis takes place in the endoplasmic reticulum, there is now considerable evidence from studies with liver that peroxisomes also play an important role in cholesterol formation [48]. It remains to be established whether in type-II cells nsL-TP is involved in peroxisomal cholesterol synthesis.

From immunological experiments on various rat tissues it has been inferred that, in common with all other tissues tested, lung contains PI-TP [15]. On the basis of cDNA analysis [17], it has been concluded that the phospholipid-transfer protein isolated from rat lung [49] is probably PI-TP. Moreover, PI-TP has been purified to homogeneity from rabbit lung [50]. Recently, PI-TP has been shown to be associated with the Golgi complex in Swiss mouse 3T3 fibroblast cells [22,51]. Similarly to that observed in yeast [52], PI-TP in mammalian cells may possibly be involved in controlling the secretory competence of the Golgi membranes. In this connection it is of interest that PI-TP is present in type-II cells (Figure 6a) and that the levels of PI-TP (Figure 2) and its mRNA (Figure 4) increase during prenatal lung development. In contrast with nsL-TP, type-II cells are not enriched in PI-TP compared with whole lung (Figure 6a). This, however, does not refute the possibility that PI-TP could be involved in secretory processes pertaining to the surfactant, such as, for example, the flow of lipids and/or proteins to the lamellar bodies.

Given the prominent presence of nsL-TP, PI-TP and PC-TP in fetal rat lung, we are currently investigating what role, if any, these proteins play in surfactant production.

The investigations described here were supported in part by the Netherlands Foundation for Chemical Research (SON) with financial aid from he Netherlands Organization of Scientific Research (NWO). We thank Dr. G. M. Helmkamp and Dr. L. R. Yarbrough and Dr. R. L. Drake for generously providing the PI-TP and malic enzyme cDNA clones respectively. We also thank Dr. J. A. J. Faber (Centre for Biostatistics, Utrecht University) for the statistical analysis of the results and Professor L. M. G. van Golde for critical reading of the manuscript.

REFERENCES

1 Wright, J. R. and Clements, J. A. (1987) Am. Rev. Respir. Dis. **135**, 426–444
2 Batenburg, J. J. (1992) Am. J. Physiol. **262**, L367–L385
3 Possmayer, F. (1988) Am. Rev. Respir. Dis. **138**, 990–998
4 Cockshutt, A. M. and Possmayer, F. (1992) in Pulmonary Surfactant, from Molecular Biology to Clinical Practice (Robertson, B., Van Golde, L. M. G. and Batenburg, J. J., eds.), pp. 339–377, Elsevier, Amsterdam
5 Askin, F. B. and Kuhn, C. (1971) Lab. Invest. **25**, 260–268
6 Chevalier, G. and Collet, A. J. (1972) Anat. Rec. **174**, 289–310
7 Engle, M. J., Van Golde, L. M. G. and Wirtz, K. W. A. (1978) FEBS Lett. **86**, 277–281
8 Teerlink, T., Van der Krift, T. P., Post, M. and Wirtz, K. W. A. (1982) Biochim. Biophys. Acta **713**, 61–67
9 Lumb, R. H., Cottle, D. A., White, L. C., Hoyle, S. N., Pool, G. L. and Brumley, G. W. (1980) Biochim. Biophys. Acta **620**, 172–175
10 Post, M., Batenburg, J. J., Schuurmans, E. A. J. M. and Van Golde, L. M. G. (1980) Biochim. Biophys. Acta **620**, 317–321
11 Pool, G. L., Bubacz, D. G., Lumb, R. H. and Mason, R. J. (1983) Biochem. J. **215**, 637–642
12 Lumb, R. H. (1989) Am. J. Physiol. **257**, L190–L194
13 Wirtz, K. W. A. (1991) Annu. Rev. Biochem. **60**, 73–99
14 Van Heusden, G. P. H., Bos, K. and Wirtz, K. W. A. (1990) Biochim. Biophys. Acta **1046**, 315–321
15 Venuti, S. E. and Helmkamp, G. M. (1988) Biochim. Biophys. Acta **946**, 119–128
16 Ossendorp, B. C., Van Heusden, G. P. H. and Wirtz, K. W. A. (1990) Biochem. Biophys. Res. Commun. **168**, 631–636
17 Dickeson, S. K., Lim, C. N., Schuyler, G. T., Dalton, T. P., Dalton, T. P., Helmkamp, G. M. and Yarbrough, L. R. (1989) J. Biol. Chem. **264**, 16557–16564
18 McHugh, K. M. and Drake, R. L. (1989) Mol. Cell. Endocrinol. **62**, 227–233
19 Tso, J. Y., Sun, X.-H., Kao, T.-H., Reece, K. S. and Wu, R. (1985) Nucleic Acids Res. **13**, 2485–2502
20 Dobbs, L. G., Gonzalez, R. and Williams, M. C. (1986) Am. Rev. Respir. Dis. **134**, 141–145
21 Teerlink, T., Van der Krift, T. P., Van Heusden, G. P. H. and Wirtz, K. W. A. (1984) Biochim. Biophys. Acta **793**, 251–259
22 Snoek, G. T., De Wit, I. S. C., Van Mourik, J. H. G. and Wirtz, K. W. A. (1992) J. Cell. Biochem. **49**, 339–348
23 Lowry, O. H., Rosebrough, N. J., Far, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
24 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
25 Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J. (1979) Biochemistry **18**, 5294–5299
26 Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, pp. 202–203, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
27 Feinberg, A. P. and Vogelstein, B. (1983) Anal. Biochem. **132**, 6–13
28 Seedorf, U. and Assmann, G. (1991) J. Biol. Chem. **266**, 630–636
29 Ossendorp, B. C., Van Heusden, G. P. H., De Beer, A. L. J., Bos, K., Schouten, G. L. and Wirtz, K. W. A. (1991) Eur. J. Biochem. **201**, 233–239
30 Mori, T., Tsukamoto, T., Mori, H., Tashiro, Y. and Fujiki, Y. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 4338–4342
31 Batenburg, J. J. and Whitsett, J. A. (1989) Biochim. Biophys. Acta **1006**, 329–334
32 Kesav, S., McLaughlin, J. and Scallen, T. J. (1992) Biochem. Soc. Trans. **20**, 818–824
33 Ossendorp, B. C. and Wirtz, K. W. A. (1993) Biochimie **75**, 191–200
34 Baum, C. L., Kansal, S. and Davidson, N. O. (1993) J. Lipid Res. **34**, 729–739
35 Levin, M. S., Pitt, A. J. A., Schwartz, A. L., Edwards, P. A. and Gordon, J. I. (1989) Biochim. Biophys. Acta **1003**, 293–300
36 Rennert, H., Amsterdam, A., Billheimer, J. T. and Strauss III, J. F. (1991) Biochemistry **30**, 11280–11285
37 Keller, G. A., Scallen, T. J., Clarke, D., Maher, P. A., Krisans, S. K. and Singer, S. J. (1989) J. Cell Biol. **108**, 1353–1361
38 Tsuneoka, M., Yamamoto, A., Fujiki, Y. and Tashiro, Y. (1988) J. Biochem. (Tokyo) **104**, 560–564
39 Van Haren, L., Teerds, K. J., Ossendorp, B. C., Van Heusden, G. P. H., Orly, J., Stocco, D. M., Wirtz, K. W. A. and Rommerts, F. F. G. (1992) Biochim. Biophys. Acta **1124**, 288–296
40 Van Amerongen, A., Van Noort, M., Van Beckhoven, J. R. C. M., Rommerts, F. F. G., Orly, J. and Wirtz, K. W. A. (1989) Biochim. Biophys. Acta **1001**, 243–248
41 Trzeciak, W. H., Simpson, E. R., Scallen, T. J., Vahouny, G. V. and Waterman, M. R. (1987) J. Biol. Chem. **262**, 3713–3717
42 Fujiki, Y., Tsuneoka, M. and Tashiro, Y. (1989) J. Biochem. (Tokyo) **106**, 1126–1131
43 Suzuki, Y., Yamaguchi, S., Orii, T., Tsuneoka, M. and Tashiro, Y. (1990) Cell Struct. Funct. **15**, 301–308
44 Van Heusden, G. P. H., Bos, K., Raetz, C. R. H. and Wirtz, K. W. A. (1990) J. Biol. Chem. **265**, 4105–4110
45 Petrik, P. (1971) J. Histochem. Cytochem. **19**, 339–348
46 Schneeberger, E. E. (1972) J. Histochem. Cytochem. **20**, 180–191
47 Schneeberger, E. E. (1972) Lab. Invest. **27**, 581–589
48 Krisans, S. K. (1992) Am. J. Respir. Cell Mol. Biol. **7**, 358–364
49 Funkhouser, J. D. (1987) Biochem. Biophys. Res. Commun. **145**, 1310–1314
50 Tsao, F. H. C., Tian, Q. and Strickland, M. S. (1992) Biochim. Biophys. Acta **1125**, 321–329
51 Snoek, G. T., Westerman, J., Wouters, F. S. and Wirtz, K. W. A. (1993) Biochem. J. **291**, 649–656
52 Cleves, A. E., McGee, T. P., Whitters, E. A., Champion, K. M., Aitken, J. R., Dowhan, W., Goebl, M. and Bankaitis, V. A. (1991) Cell **64**, 789–800

Received 19 July 1993/1 September 1993; accepted 23 September 1993