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# Mice without phosphatidylcholine transfer protein have no defects in the secretion of phosphatidylcholine into bile or into lung airspaces

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**ABSTRACT** **Phosphatidylcholine transfer protein (Pc-tp) is a highly specific carrier of phosphatidylcholine (PC) without known function. Proposed functions include the supply of PC required for secretion into bile or lung air space (surfactant) and the facilitation of enzymatic reactions involving PC synthesis or breakdown. To test these functions, we generated knock-out mice unable to make Pc-tp. Remarkably, these mice are normal and have no defect in any of the postulated Pc-tp functions analyzed. The lipid content and composition of the bile, as well as lung surfactant secretion and composition, of *Pc-tp* (−/−) mice, is normal. The lack of a Pc-tp contribution to biliary lipid secretion is in agreement with our finding that Pc-tp is down-regulated in adult mouse liver: whereas Pc-tp is abundant in the liver of mouse pups, Pc-tp levels decrease > 10-fold around 2 wk after birth, when bile formation starts. In adult mice, Pc-tp levels are high only in epididymis, testis, kidney, and bone marrow-derived mast cells. Absence of Pc-tp in bone marrow-derived mast cells does not affect their lipid composition or PC synthesis and degradation. We discuss how PC might reach the canalicular membrane of the hepatocyte for secretion into the bile, if not by Pc-tp.**

Phosphatidylcholine transfer protein (Pc-tp) is a 28-kDa protein originally isolated from bovine liver cytosol. *In vitro* this protein can accelerate intermembrane transfer of phospholipids in an energy-independent way (1). In contrast to other phospholipid transfer proteins, Pc-tp is highly specific for phosphatidylcholine (PC) (2). Even minor alterations of the phosphorylcholine headgroup greatly diminish or eliminate transfer by Pc-tp.

Despite its discovery 30 years ago and its intensive biochemical characterization, remarkably little is known about the biological function of Pc-tp. On the basis of its tissue distribution and binding specificity, a variety of functions have been proposed. A widely accepted putative function for Pc-tp is the delivery of PC to the PC-translocator, Mdr2 P-glycoprotein (Pgp), in the bile canalicular membrane (3–8). Pc-tp is thought to obtain its substrate PC at the place of PC synthesis, the cytosolic leaflet of the endoplasmic reticulum and the Golgi apparatus (9), and shuttle the lipid as a monomer to the cytosolic leaflet of the canalicular membrane of the hepatocyte, where the PC translocator can translocate PC for delivery into bile (4).

Another role proposed for Pc-tp is in the secretion of lung surfactant. Pc-tp mRNA levels are relatively high in the surfactant producing type II cells (10) and increase in the fetal mouse and rat lung in the period of accelerated surfactant formation (11–13). Hence, Pc-tp could be involved in the PC transfer between the endoplasmic reticulum and the surfactant containing lamellar bodies or in PC synthesis in the type II cells (for review see ref. 14).

Several other functions have been proposed for Pc-tp: determining the fatty acid specificity of the PC present in bile (7) or providing help by delivery or removal of PC in various enzymatic reactions, such as hydrolysis of PC by phospholipases (15), or in the synthesis of sphingomyelin (16) or PC (17).

To resolve the biological function of Pc-tp, we disrupted the *Pc-tp* gene in mice. Unexpectedly, none of the putative functions attributed to Pc-tp appear to be defective in mice without Pc-tp.

## MATERIALS AND METHODS

**Cell Culture.** Embryonic stem cells, clone E14, were grown on mitotically inactivated mouse embryonic fibroblasts in Glasgow MEM supplemented with 10% FCS/1× nonessential amino acids/2 mM L-glutamine/1 mM sodium pyruvate/0.1 mM β-mercaptoethanol/$10^3$ U ESGRO-leukemia inhibitory factor/ml (GIBCO BRL). During G418 selection, the embryonic stem cells were grown in 60% buffalo rat liver cells conditioned medium with $10^3$ units ESGRO-leukemia inhibitory factor/ml (18).

**Isolation of Mouse and Human cDNAs.** Two independent rapid amplification of cDNA ends (5′RACE) were performed on 1 μg mouse RNA according to the manufacturer's instructions (GIBCO BRL) to complete the 5′ sequence information lacking from the mouse Pc-tp clone isolated by Geijtenbeek *et al.* (12). Both fragments were sequenced, and missing coding sequence was ligated to the 3′ part of the mouse cDNA.

The human cDNA was sequenced from the clone aa030013 obtained from the I.M.A.G.E. Consortium library (19). Both cDNAs resulted in an ORF of 624 bp. The cDNA sequences have been deposited in GenBank under database accession nos. AF151638 (human) and AF151639 (mouse).

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Abbreviations: PC, phosphatidylcholine; BMMC, bone marrow-derived mast cells; TUDC, tauroursodeoxycholate; GPC, glycerophosphocholine.

Database deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF151638 for huPC-TP and AF151639 for muPc-tp).

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**Disruption of the *Pc-tp* Gene by Homologous Recombination.** From a lambda EMBL3 genomic 129/Ola DNA library (20) four independent *Pc-tp* clones of 10.5 kb were isolated and characterized by Southern blotting to map the intron-exon boundaries. These clones contained 78% of the *Pc-tp* coding sequence in 5 exons B, C, D, E, and F. A sixth exon A was found to be >3.5 kb upstream of exon B and not present in these genomic clones.

To disrupt the *Pc-tp* gene, we made a targeting construct as depicted in Fig. 2*A*. In the construct, made with isogenic DNA, 60% of the *Pc-tp* gene was replaced by the pPgk-Neomycin-bpA cassette in the reverse orientation. A 4.9-kb Asp-718 fragment containing the second *Pc-tp* exon was ligated behind the pPgk-Neomycin-bpA cassette in PBS-KS. Subsequently the 3′ arm, a 1.9-kb *Xba*I fragment upstream of the sixth exon, was ligated in front of the pPgk-Neomycin-bpA cassette. One hundred micrograms of the construct was linearized and electroporated into $5 \times 10^7$ embryonic stem cell line (E14 ES) with a Bio-Rad GenePulser at 3 $\mu$F and 0.8 kV per 0.4 cm. G418-resistant clones were tested for homologous recombination by blotting the genomic DNA and hybridization with the 3′ and the 5′ *Pc-tp* probe. Of 650 G418-resistant clones, three were identified as positives giving the expected wild-type bands of 5 kb (3′ probe) and 10 kb (5′ probe) and the mutated fragments of 12 kb (3′ and 5′ probe).

**Protein Analysis.** Tissue was homogenized in HBT buffer (0.25 M sucrose/10 mM Tris·HCl, pH7.35/1 mM EDTA/1 mM PMSF/1 $\mu$g/ml leupeptin/1 $\mu$g/ml pepstatin/1 $\mu$g/ml aprotinin), and cells were disrupted by 20 strokes through a 23-G needle. Nuclei, mitochondria, and membranes were removed by centrifugation with $1{,}000 \times g$, $10{,}000 \times g$, and $100{,}000 \times g$, respectively. Protein concentration was determined by using a Bio-Rad protein assay. Cytosolic proteins were separated in a 12.5% (wt/vol) SDS/PAGE gel. After electroblotting to nitrocellulose in 25 mM Tris/200 mM glycine/20% methanol, Pc-tp was visualized by staining with the affinity purified polyclonal antibody Ab6221, followed by the enhanced chemiluminescence procedure (Amersham Pharmacia). The phosphatidylinositol transfer proteins were detected with the polyclonal antibody Ab9026 recognizing both the Pi-tp$\alpha$ and the Pi-tp$\beta$ form (21), and the nonspecific lipid transfer protein (Nsl-tp) was detected with the polyclonal antibody Ab8723.

**Assay for Pc-tp Activity by Using a Fluorescent Substrate.** Membrane-free cytosol fractions from 4-day-old pup livers were adjusted to pH 5.1 and centrifuged to remove denatured proteins. After readjustment to pH 5.5 and dialysis against 10 mM sodium acetate (pH 5.5), samples were separated in 50 fractions on a Resource S (Amersham Pharmacia) in the same buffer. PC transfer activity was determined by measuring the fluorescence appearing after transfer of 2-pyrenyl-acyl-PC from a donor vesicle containing the quencher trinitrophenyl-phosphatidylethanolamine (PE) to an acceptor vesicle (22). The donor vesicles consisted of 2-pyrenyl-decanoyl-PC/egg PC/phosphatidic acid (PA)/trinitrophenol-PE (10:70:10:10 mol %) and the acceptor vesicles of egg PC and PA (95:5 mol %). Pi-tp$\alpha$, Pi-tp$\beta$, and Nsl-tp transfer activity were measured under identical conditions with 2-pyrenyl-decanoyl-PC but also with donor vesicles consisting of 2-pyrenyl-decanoyl-Pi/egg PC/trinitrophenol-PE (10:80:10 mol %).

**Bile Composition.** Two-month-old male mice were anesthetized by i.p injection of 1 ml Hypnorm (fentanyl/fluanisone) and 10 mg diazepam per kg body weight. To collect bile, the abdomen was opened and the gallbladder was cannulated after distal ligation of the common bile duct. The body temperature was maintained by placing the mice on a thermostatted heating pad and covering them with a piece of foil. Bile samples were collected and frozen at −80°C. Bile flow was determined gravimetrically assuming a density of 1 g/ml for bile. Choline content of PC and sphingomyelin was determined enzymatically with phospholipase D and choline oxidase (23). Cholesterol was assayed enzymatically with cholesterol oxidase (24). Total bile salt concentration was measured spectrophotometrically with 3$\alpha$-hydroxysteroid dehydrogenase (25). Bile formation and lipid secretion during i.v. infusion of tauroursodeoxycholate (TUDC): bile was collected from the cannulated gallbladder to deplete endogenous bile salts and analyzed as described above. After 90 min, an increasing concentration of TUDC was infused via a tail vein from 600 nmol TUDC/min per 100 g to 2,400 nmol/min per 100 g as before (26).

**Bronchoalveolar Lavage.** Mice were lightly anesthetized by inhalation of isoflurane, and endotoxin (from *Escherichia coli* serotype O111B4, Sigma) in 50 $\mu$l isotonic saline was inoculated intranasally. Control mice were inoculated intranasally with 50 $\mu$l of isotonic saline only. At 6 hr after inoculation, mice were anesthetized with Hypnorm and midazolam. To analyze the surfactant, the bronchoalveolar lavage fluid was obtained. The trachea was exposed through a midline incision and cannulated with a sterile 22G Abbocath-T catheter (Abbot). Bronchoalveolar lavage was performed by instilling two 0.5-ml aliquots of sterile isotonic saline. Lavage fluid (0.8–1.0 ml per mouse) was retrieved, spun at $750 \times g$ for 5 min at 4°C, and supernatants were stored at −80°C until lipid analysis.

**Labeling and Separation of Lipids.** Cells ($1 \times 10^6$) were preincubated in Tyrode's salt solution for 1 hr at 37°C to reduce cellular choline and labeled with [$^{14}$C]choline chloride (1 $\mu$Ci/ml medium) or with [$^{14}$C]ethanolamine (0.2 $\mu$Ci/ml medium) at 37°C. Lipids were extracted according to Bligh and Dyer (27). The organic (lower) phase was dried under $N_2$, and lipids were applied to silica-60 TLC plates (Merck) with chloroform/methanol (1:1). The lipids were separated in one dimension in chloroform/methanol/25% ammonia (65:35:4). For analysis of the water (upper) phase, the samples were dried in a Speed-Vac (Savant, Hicksville, NY) applied to silica-60 TLC plates with water/methanol (2:1) and separated in water/methanol/ammonia 25% (5:5:1). The TLC plates were exposed to imaging screens (Fuji), and radiolabeled spots were quantified.

Fatty acid composition in PC was determined by HPLC analysis of the intact molecular species as described (28). Identification of molecular species was performed by on-line electrospray mass spectrometry as described (29).

**Isolation and Incubation of Bone Marrow-Derived Mast Cells (BMMCs).** Three independent samples of BMMCs were prepared and cultured as described (30). Cultures of wild-type and *Pc-tp* (−/−) cells were indistinguishable in morphology and growth characteristics. To induce leukotriene synthesis, cells were incubated for 20 min as described (31) with 0.2 $\mu$M A23187 calcium ionophore at 37°C. For leukotriene analysis, the reaction was stopped by the addition of 4 vol of ice-cold ethanol. Protein aggregates were removed by centrifugation, and leukotrienes were extracted and separated by HPLC as before (32). For lipid analysis and labeling studies, the reaction was stopped by Bligh and Dyer extraction (27).

## RESULTS

**Characterization of Mouse and Human cDNAs.** We determined the sequence of the mouse and human Pc-tp cDNAs. The corresponding proteins are highly homologous to rat (6) and bovine (12, 33) Pc-tp (Fig. 1). The hydrophobic domain involved in the binding of the *sn2* fatty acyl chain ($V^{171}$-$D^{177}$) (34) is 100% identical in all four proteins, but also the predicted antiparallel $\beta$ sheet $Q^{182}$-$W^{190}$ and the other sequences adjacent to the binding domain appear to be conserved, which suggests that the entire region is part of the highly specific lipid-binding site. Similarly, $Y^{54}$ proposed to be at the surface of the protein yet close to the lipid-binding site (34) is conserved in all four proteins. As the $C^{93}$ is not

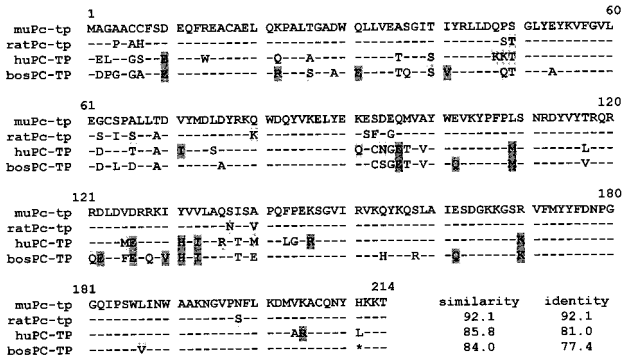


FIG. 1. Sequence alignment of the deduced amino acid sequence of mammalian Pc-tps given in one-letter code. Amino acid sequences of mouse (muPc-tp), rat (ratPc-tp), human (huPC-TP), and bovine (bosPC-TP) Pc-tp deduced from the corresponding cDNAs. A dash indicates an amino acid identical to the mouse consensus, and an asterisk indicates a deletion. Highly similar amino acids are shaded in dark gray, and amino acids with a lower similarity are shaded in light gray. The overall similarity and identity of the PC-TPs to mice Pc-tp are indicated.

conserved, we conclude that the postulated disulfide bond with $C^{207}$ is not essential for the stabilization of the protein.

We found no protein homologous to Pc-tp by searching in GenBank, nor did any additional band come up after low-stringency hybridization of genomic Southern blots with *Pc-tp* cDNA (data not shown). These results indicate that the murine genome contains only a single gene for Pc-tp.

**Generation and Characterization of *Pc-tp* Knock-Out Mice.** Chimeric mice were obtained by injection of two independently isolated *Pc-tp* (+/−) embryonic stem cell clones (T5 and M8) into blastocysts following standard procedures (35). Both clones resulted in chimeric mice that transmitted the mutated *Pc-tp* allele through the germline. To obtain homozygous *Pc-tp* (−/−) mice, heterozygous mice of both strains (T5 and M8) were separately inbred. Southern blot analysis of 307 tails gave 25.1% wild-type (+/+), 49.2% heterozygous (+/−), and 25.7% homozygous *Pc-tp* (−/−) mice, indicating that the disruption of the *Pc-tp* gene does not significantly alter the viability of (+/−) and (−/−) mice. The (−/−) and (+/−) mice could not be distinguished from (+/+) mice by any exterior abnormalities or mortality rate up to 7 mo of age ($n$ = 6). Extensive histological analysis of 6-mo-old males and females revealed no substantial differences between *Pc-tp* (+/+) and *Pc-tp* (−/−) mice. Homozygous (−/−) mice, when inbred, are fertile and give rise to viable progeny. The litters are of comparable size to wild type. The heterozygous, homozygous, and wild-type mouse stocks were maintained as a cross of FVB and 129/Ola (50%/50%), as the 129/Ola mice breed poorly.

The absence of an obvious phenotype in the knock-out mice raised the question whether the gene disruption had been successful. Fig. 2 shows that it is. Southern blot analysis with 5′ and 3′ probes resulted in the expected bands (Fig. 2*B*). Fig. 2*C* shows that disruption of the *Pc-tp* gene resulted in a decrease in Pc-tp protein of approximately 50% in the (+/−) and in its complete absence in the (−/−) mouse. The total PC transfer activity in pup-liver cytosol of Pc-tp (−/−) mice decreased 35–40% (not shown). To identify the remaining activity, we separated the proteins on a Resource S column. We found no increased amounts of other transfer proteins, such as the Nsl-tp and the phosphatidylinositol transfer proteins $\alpha$ and $\beta$ (Pi-tp $\alpha/\beta$) in the cytosol of the *Pc-tp* (−/−) mice, as measured by their transfer activity *in vitro* and by ELISA by using specific antibodies (data not shown). Fig. 2*D* shows that PC transfer activity resulting from Pc-tp was absent from the *Pc-tp* (−/−) pup liver.

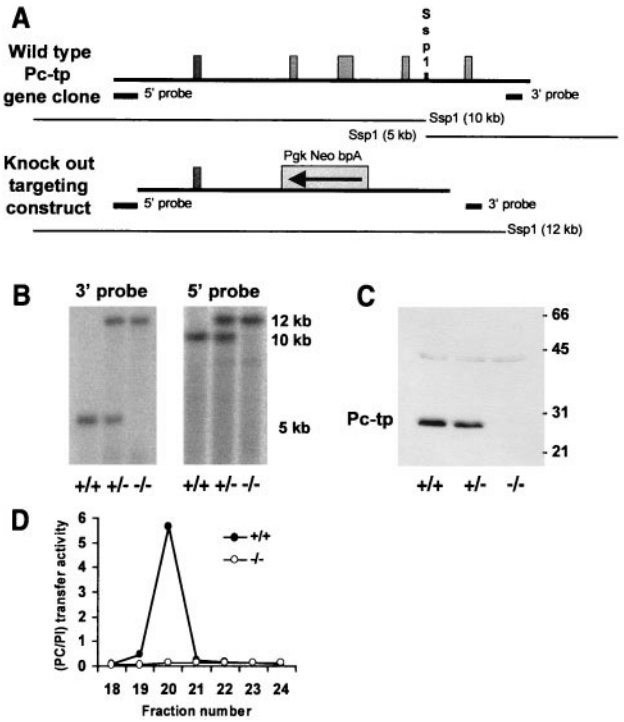


FIG. 2. Targeting of the *Pc-tp* gene by homologous recombination. (*A*) Structure of the five *Pc-tp* exons in the isolated genomic clone and the targeting vector with the Neomycin cassette flanked by 4.9 kb and 1.9 kb of 5′ and 3′ arms, respectively. The locations of the 5′ and the 3′ probes flanking the homologous arms are indicated, as are the expected fragments after *Ssp*I digestion. (*B*) Southern blot analysis of *Ssp*I digest of mouse tail DNA hybridized with either the 5′ or the 3′ probe and showing the expected genomic fragments in *Pc-tp* (+/+), *Pc-tp* (+/−), and *Pc-tp* (−/−) mice. (*C*) Western blot analysis with the polyclonal antibody Ab6221 detected as in *Materials and Methods* showing a decrease of Pc-tp in the cytosol of an 8-day-old *Pc-tp* (+/−) pup, and the complete absence of Pc-tp in a *Pc-tp* (−/−) pup (20 $\mu$g protein/lane). (*D*) Pc-tp activity in pup liver cytosol fractions from the Resource S column. PC and phosphatidylinositol (PI) transfer activity were determined as in *Materials and Methods*. By presenting the ratio of PC and PI transfer activity, the contribution of Pi-tp and Nsl-TP to the PC transfer activity was corrected for.

**PC Secretion into Bile.** We have previously shown that mice unable to make the canalicular PC translocator, the Mdr2 P-gp, develop cholestatic liver disease, because they are unable to secrete PC into their bile (4). Because it was thought that Pc-tp is essential for transporting PC to the canalicular membrane to supply the Mdr2 P-gp with a constant source of PC, we expected a similar cholestatic liver disease in the *Pc-tp* knock-out mice. The mice were fine, however. To test whether there was any effect of the absence of Pc-tp on PC secretion, we collected bile for 2 hr from 2-mo-old male mice by cannulating the gallbladder after distal ligation of the common bile duct. Compared with wild-type mice [2.9 ± 0.6 $\mu$mol/2 h per 100 g ($n$ = 2)], secretion of choline containing phospholipids into bile was not diminished in *Pc-tp* (+/−) [3.0 ± 0.1 $\mu$mol/2 h per 100 g ($n$ = 2)] or *Pc-tp* (−/−) mice [3.0 ± 0.5 $\mu$mol/2 h per 100 g ($n$ = 2)]. Also other bile parameters, such as bile flow, bile salt output, and cholesterol output were not affected by disruption of the *Pc-tp* gene.

In an independent experiment, we infused the mice with TUDC via a tail vein to test PC secretion at maximal capacity. The *Pc-tp* (−/−) mice responded with increased PC secretion, just like wild-type mice (Fig. 3). To check whether the phospholipid composition in the (−/−) bile was altered, we analyzed the bile by HPLC. No differences in lipid content and lipid composition were found. In both mice, over 95% of total phospholipid was PC (not shown).

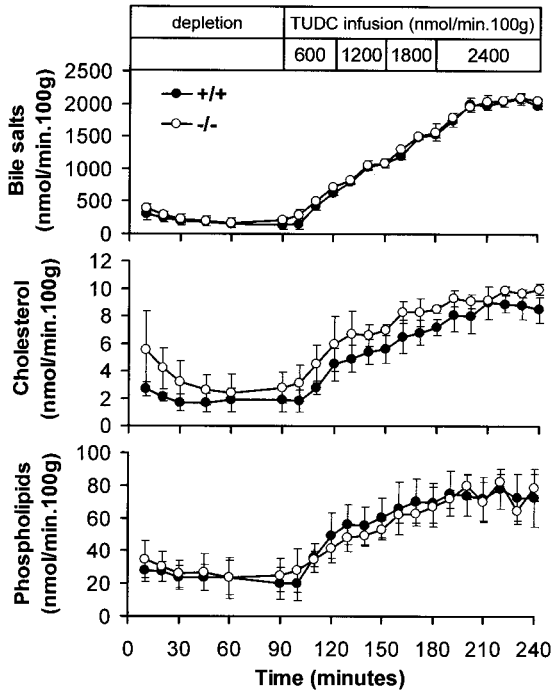


FIG. 3. Analysis of bile from *Pc-tp* (+/+) and (−/−) mouse strains during i.v. infusion of TUDC. Wild-type (●) and *Pc-tp* (−/−) mice (○) were cannulated in the gallbladder; bile was sampled for 90 min to deplete the bile salt pool and then TUDC was infused via a tail vein at the indicated increasing rates. Data represent means of three mice (±SD). (*Top*) Bile salt secretion; (*Middle*) cholesterol secretion; (*Bottom*) phospholipid secretion.

Pc-tp has the highest affinity for PC with a fatty acid composition resembling that of bile PC. This led to the suggestion that Pc-tp determines the selection of the PC species characteristic for bile (7). Table 1*A* shows, however, that the fatty acids in bile PC of *Pc-tp* (−/−) mice are indistinguishable from those of wild-type mice.

**Surfactant Production in Lung.** PC is a major component of lung surfactant, and it has been suggested that Pc-tp is required for surfactant formation or secretion. Indeed, the Pc-tp level peaks in the fetal mouse and rat lung in the period of accelerated surfactant formation (11–13). However, we did not notice any respiratory distress in the *Pc-tp* null mice after birth or later. Electron microscopy analysis of the adult lung of *Pc-tp* (−/−) mice shows no morphological abnormalities either. Lamellar bodies look typical, and surfactant is secreted normally (Fig. 4). To induce maximal PC secretion in the lung, the mice were instilled with lipopolysaccharides. Even under these stressed conditions, the null mice made normal amounts of surfactant and there was no change in the fatty acid composition of the PC species present in surfactant (Table 1*B*).

Table 1. PC species in mol percent of total PC present in bile and in lung surfactant

| PC species | mol % *Pc–tp*(+/+) | *Pc–tp*(−/−) |
|---|---|---|
| | In bile | |
| $C_{16:0}$–$C_{16:1}$ | 1.5 ± 0.8 | 0.4 ± 0.4 |
| $C_{16:0}$–$C_{18:1}$ | 10.1 ± 0.4 | 5.1 ± 2.6 |
| $C_{16:0}$–$C_{18:2}$ | 60.0 ± 3.5 | 61.9 ± 2.8 |
| $C_{16:0}$–$C_{20:3}$ | 0.6 ± 0.4 | 0.1 ± 0.1 |
| $C_{16:0}$–$C_{20:4}$ | 13.0 ± 1.0 | 12.5 ± 0.8 |
| $C_{16:0}$–$C_{22:6}$ | 9.3 ± 0.9 | 9.8 ± 0.1 |
| $C_{16:1}$–$C_{18:2}$ | 1.0 ± 1.0 | 1.7 ± 0.9 |
| $C_{18:0}$–$C_{18:2}$ | 4.2 ± 0.3 | 8.3 ± 2.6 |
| $C_{18:0}$–$C_{20:4}$ | 0.3 ± 0.2 | 0.3 ± 0.1 |
| | In lung surfactant | |
| $C_{16:0}$–$C_{16:0}$ | 45.4 ± 0.9 | 41.6 ± 5.0 |
| $C_{16:0}$–$C_{16:1}$ | 10.1 ± 0.2 | 8.8 ± 0.7 |
| $C_{16:0}$–$C_{18:1}$ | 6.5 ± 0.7 | 5.7 ± 0.8 |
| $C_{16:0}$–$C_{18:2}$ | 26.6 ± 0.8 | 25.4 ± 2.2 |
| $C_{16:0}$–$C_{20:4}$ | 6.6 ± 0.5 | 5.7 ± 0.6 |
| $C_{16:0}$–$C_{22:6}$ | 1.4 ± 0.0 | 1.4 ± 0.2 |
| $C_{18:0}$–$C_{18:1}$ | 0.3 ± 0.2 | 0.1 ± 0.1 |
| $C_{18:0}$–$C_{22:6}$ | 2.6 ± 0.7 | 3.1 ± 1.7 |

**Tissue Distribution and Intracellular Localization of Pc-tp.** Because the *Pc-tp* (−/−) mice did not give the expected phenotype, we checked the reported tissue distribution of Pc-tp in the tissues of 2- to 3-mo-old mice by using Western blotting with an antibody specific for Pc-tp. For most tissues, the murine Pc-tp levels (Table 2) nicely correlate with data reported earlier for the rat (13). Only liver shows an unexpectedly low level of Pc-tp in adult mice, in marked contrast to the high Pc-tp levels detected in pup livers (Fig. 2*C*). Fig. 5 shows that Pc-tp is high in the first week after birth but falls to a hardly detectable level within 2 wk after birth. Because hematopoiesis takes place in the pup liver, and because Pc-tp is high in BMMCs (Table 2), it seemed possible that Pc-tp is not present in the hepatocytes but in the blood cells maturing in the pup liver. Immunohistochemical analysis showed, however, that Pc-tp is present in the hepatocytes (Fig. 6*C*).

Whereas the Pc-tp in the testis is present in all cells involved in spermatogenesis, the Pc-tp in the epididymis is restricted to the epithelial cells covering the sperm ducts (Fig. 6*E*) and absent from mature spermatozoa. Previous work has suggested that Pc-tp is a cytosolic protein (13), and our light microscopical pictures are compatible with this localization of Pc-tp.

**PC Metabolism.** To test whether Pc-tp has an effect on the synthesis of PC, we labeled BMMCs and mouse pup livers with [$^{14}$C]choline. The BMMCs from *Pc-tp* (+/+) and *Pc-tp* (−/−) mice were grown in the presence of IL-3 for 4 wk to get >98% pure primary cells. We found no effect of the presence of Pc-tp on the labeling kinetics in these cells (not shown). Also the total phospholipid composition under steady-state conditions and the different PC species did not differ significantly between *Pc-tp* (+/+) and (−/−) cells (not shown). This makes it very unlikely that Pc-tp plays an indispensable role in PC synthesis, hydrolysis, reacylation, or even in sphingomyelin synthesis. No differences in labeling kinetics or phospholipid composition were found in radiolabeled homogenized pup livers either (not shown).

**Leukotriene Synthesis.** Because leukotriene synthesis and secretion is a major activity of mast cells, Pc-tp might have a role in the supply of the arachidonic acid containing PCs to the

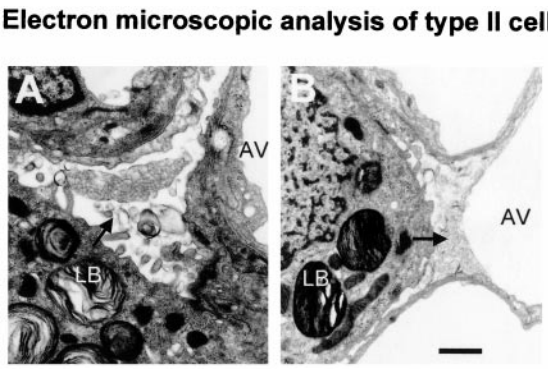


FIG. 4. Electron microscopic analysis of type II cells. (*A*) *Pc-tp* (+/+) lung; (*B*) Pc-tp (−/−) lung. LB, lamellar bodies; AV, alveolar spaces; arrows point to surfactant secreted from the type II cells. Bar = 1 μm.

Table 2. Tissue distribution of mouse Pc–tp determined with an antibody against Pc–tp

| | | | |
|---|---|---|---|
| Stomach | − | Skeletal muscle | +/− |
| Duodenum | − | Hart muscle | +/− |
| Jejunum | +/− | Spleen | + |
| Ileum | +/− | Thymus | − |
| Caecum | + | Central nervous system | − |
| Colon | + | Skin | − |
| Liver | +/− | Fat tissue | − |
| Liver (pup) | +++++ | Testis | +++ |
| Bladder | + | Epididymis | ++++ |
| Kidney | ++ | Secondary sex glands | − |
| Adrenal | +/− | Milk gland | +/− |
| Lung | + | Uterus | +/− |
| | | BMMC | +++++ |

Total protein was isolated from the different tissues, and analyzed by Western blotting with the polyclonal antibody Ab6221 as in *Material and Methods*. The relative expression levels are indicated by +/− (hardly detectable) to +++++ (highest expression).

$Ca^{2+}$ sensitive phospholipase A2 (PL-$A_2$). On stimulation of this enzyme with $Ca^{2+}$, arachidonic acid is liberated, and this is converted into the leukotrienes that are released from the BMMCs (31). Three independent wild-type and three independent *Pc-tp* (−/−) BMMC cell cultures were labeled to equilibrium with [$^{14}$C]choline for 72 hr. We then stimulated PC hydrolysis with the $Ca^{2+}$ ionophore A23187 and analyzed the formation of lyso-PC and the synthesis and secretion of leukotrienes. The absence of Pc-tp had no effect on PC hydrolysis (not shown) or on leukotriene synthesis. In both *Pc-tp* (+/+) and *Pc-tp* (−/−) BMMCs, we observed a clear increase of dihydroxyeicosatetraenoic acids (DiHETEs) and leukotriene-$B_4$s ($LTB_4$s) after stimulation. The amounts of $LTB_4$ ($105 \pm 28/10^7$ cells) and 5,6 DiHETEs ($20 \pm 7/10^7$ cells) in wild-type cells were similar to the amounts in *Pc-tp* (−/−) cells, $101 \pm 42$ and $17 \pm 4/10^7$ cells, respectively.

## DISCUSSION

Previous work by us and others has led to the idea that Pc-tp plays an essential role in providing PC for secretion into the bile. The results presented here show that this idea is wrong. Maximal rates of PC secretion into bile are unaltered in our *Pc-tp* (−/−) mice. It is improbable that our mice compensate for the absence of Pc-tp by overproducing other PC transport proteins, because we found no increase in the hepatic levels of the other known phospholipid transfer proteins, Nsl-TP and Pi-tp $\alpha/\beta$, in the *Pc-tp* (−/−) mice. The absence of an effect of Pc-tp on PC secretion is in agreement with the regulation of Pc-tp levels in normal mouse liver: around the time that bile secretion begins, there is a precipitous fall in the level of hepatic Pc-tp.

Taken together, these data prove that Pc-tp cannot play a major role in transporting PC from its place of synthesis in the endoplasmic reticulum to the canalicular membrane of the hepatocyte. How this lipid does get to the canalicular membrane is unclear.

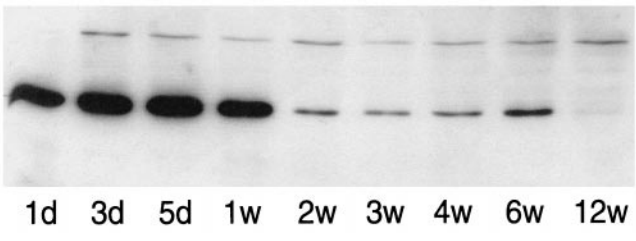


FIG. 5. Pc-tp levels in mouse liver as a function of time after birth. Equal amounts of protein from liver cytosol isolated from *Pc-tp* (+/+) mice of different age were loaded on the gel. Pc-tp expression was detected by incubating the Western blot with the polyclonal antibody Ab6221 and visualized with the enhanced chemiluminescence procedure as in *Materials and Methods.*

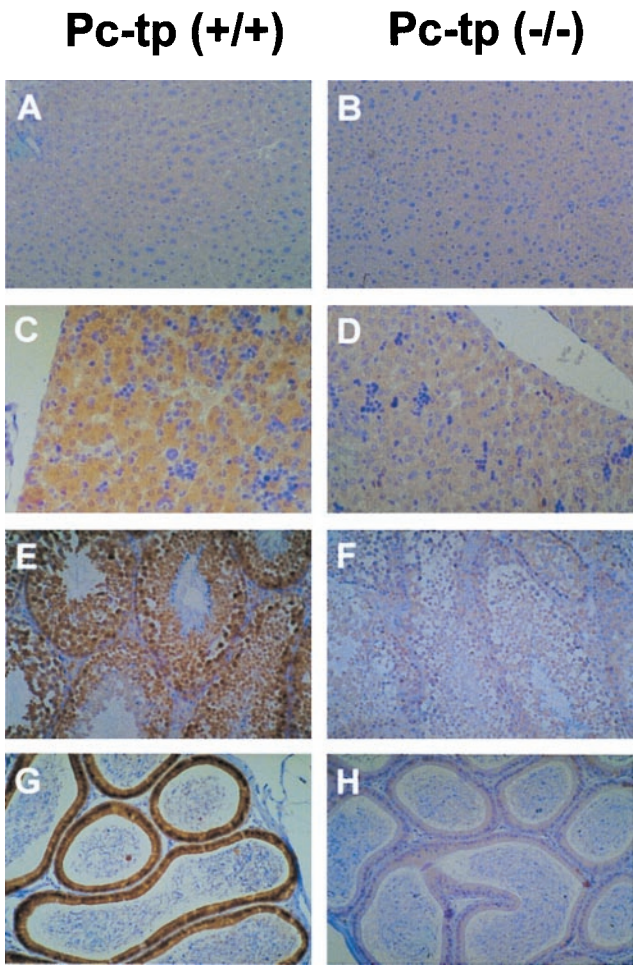


FIG. 6. Immunohistochemical detection of Pc-tp in sections of formaldehyde fixed paraffin sections. Sections were incubated with a 1:50 dilution of the polyclonal antibody Ab6221 for *A–D* and Ab6222 for *E–H* as before (36). (*A*) Section of an 8-wk-old *Pc-tp* (+/+) liver; (*B*) section of an 8-wk-old *Pc-tp* (−/−) liver; (*C*) section of a 2-day-old *Pc-tp* (+/+) liver; (*D*) section of a 2-day-old *Pc-tp* (−/−) liver; (*E*) section of an 8-wk-old *Pc-tp* (+/+) testis; (*F*) section of an 8-wk-old *Pc-tp* (−/−) testis; (*G*) section of an 8-wk-old *Pc-tp* (+/+) epididymis; and (*H*) section of an 8-wk-old *Pc-tp* (−/−) epididymis.

We see three possibilities: (*i*) monomeric PC transport by a transport protein that remains to be identified; (*ii*) vesicular transport directly to the canalicular membrane. This alternative has been proposed on the basis of inhibitor experiments (37–39) but seemed unlikely because of the absolute requirement for a functional *Mdr2* gene (4). This gene encodes a P-glycoprotein (Pgp) that can translocate PC from the inner leaflet to the outer leaflet of the plasma membrane (4, 40, 41). In the absence of the Mdr2 Pgp PC secretion is abolished (4). If PC would arrive at the canalicular membrane by vesicular transport, one would expect that 50% of the PC is delivered to the outer leaflet and directly available for entry into bile without intervention of the Mdr2 Pgp. However, no PC secretion into bile was found in the Mdr2 Pgp (−/−) mice (see ref. 4). It is possible, however, that the Mdr2 Pgp is not only actively flipping PC from inner to outer leaflet, but also destabilizing the outer leaflet in the process. This destabilization by the increased lateral pressure in the exoplasmic leaflet of the canalicular membrane could facilitate the extraction of PC from the membrane by bile salts. Indeed, the characteristic vesicular structures that bulge from the canalicular membrane

into the bile (42) depend on the presence of a functional Mdr2 Ppg (43). Even PC directly delivered to the outer leaflet of the canalicular membrane might therefore not be available for secretion unless the Mdr2 Pgp is active. (*iii*) Secretion of plasma (lipo)proteins requires intensive vesicular transport to the basolateral membrane of the hepatocyte (44, 45). The PC deposited in the basolateral part of the outer plasma membrane leaflet in this way cannot reach the canalicular membrane, as lateral diffusion is prevented by the tight junctions (46). These junctions do not block lateral diffusion in the inner leaflet of the plasma membrane, however. In this way the PC would reach the Mdr2 Pgp in the canalicular membrane to be flipped to the outer leaflet.

Alternatives *i* and *iii* provide the best explanation for the observation that bile PC differs from hepatocyte PC in fatty acid composition (47, 48). This difference could be caused either by selective transport, or by selective translocation by the Mdr2 Pgp. Alternative *ii* can explain this difference only by postulating selective vesicular transport of bile-specific PC. This seems an unlikely option.

In adult mice, we found only high levels of Pc-tp in epididymis, testis, and BMMCs, and somewhat lower levels in kidney. Especially striking is the intense staining of the epithelial cells covering the sperm ducts in the epididymis. These cells secrete high amounts of glycerophosphocholine (GPC), which is involved in the capacitation of the sperm. GPC is made from precursor PC by removing the two fatty acids. This raised the question whether Pc-tp might be involved in GPC formation, but we have found no indication for this. The fertility of the male *Pc-tp* (−/−) mice was not altered, nor was the GPC content of the epididymis (not shown). The kidney also produces significant amounts of GPC to regulate the osmolarity of the epithelial cells facing the distal part of the tubuli. No morphological abnormalities were found by lightmicroscopical analysis of the *Pc-tp* (−/−) kidneys. So Pc-tp does not contribute to the production of GPC, or compensation occurs when this protein is lacking.

Our conclusion is that the physiological function of Pc-tp remains unknown. Our analysis of *Pc-tp* (−/−) mice appears to eliminate all functions previously proposed and additional functions that could follow from its transport specificity and tissue distribution. Further analysis of these mice that are available to other investigators, with no strings attached, should help to find the physiological function of Pc-tp.

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