Functional studies on the phosphatidylcholine transfer protein

Functionele studies aan het fosfatidylcholine transport eiwit

(met een samenvatting in het Nederlands)

Proefschrift

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Calvin: I've decided not to go to school this fall. I don't need an education. I don't need to develop skills. It's too much trouble.

Hobbes: How are you going to make it in the world if you don't know anything and you don't have any skills ?!

Calvin: I'll go on talk shows and hype myself.

voor 's pap en 's mam

Contents

Abbreviations

BCIP	=	5-bromo-4-chloro-3-indoyl-phosphate *p*-toluidine salt
BMMCs	=	bone marrow-derived mast cells
C16:0	=	palmitic acid
C18:0	=	stearic acid
C18:1	=	oleic acid
C20:4	=	arachidonic acid
CDP	=	cytidine diphosphate
CMP	=	cytidine monophosphate
CLSM	=	confocal laser scanning microscopy
CV	=	cone voltage
DMEM	=	Dulbecco's-modified Eagles medium
DMSO	=	dimethyl sulphoxide
DTT	=	dithiotreitol
ESI-TOF	=	electrospray ionization time-of-flight mass spectrometry
EYFP	=	enhanced yellow fluorescent protein
FAB-MS	=	fast atom bombardment mass spectrometry
FCS	=	foetal calf serum
FFA	=	free fatty acids
GAR-AP	=	goat-anti-rabbit IgG conjugated with alkaline phosphatase
GPC	=	glycerophosphocholine
HDL	=	high-density lipoprotein
HPLC	=	high performance liquid chromatography
HP-TLC	=	high performance thin layer liquid chromatography
IEP	=	isoelectric point
IMAC	=	immobilized metal ion affinity chromatography
IPTG	=	isopropyl-β-D-thiogalactopyranoside
LB medium	=	Luria-Bertani medium
LDL	=	low-density lipoprotein
Mw	=	molecular weight
βME	=	β-mercaptoethanol
NCS	=	newborn calf serum
nsL-TP	=	non-specific transfer protein
NBT	=	*p*-nitro blue tetrazolium chloride
PA	=	phosphatidic acid
PBS	=	phosphate-buffered saline

PC	=	phosphatidylcholine
PC-TP	=	phosphatidylcholine transfer protein
PE	=	phosphatidylethanolamine
PG	=	phosphatidylglycerol
PI	=	phosphatidylinositol
PI-TP	=	phosphatidylinositol transfer protein
PMA	=	phorbol 12-myristate 13-acetate
PPAR	=	peroxisome proliferator-activated receptor
PUFA	=	polyunsaturated fatty acid
RT	=	room temperature
RXR	=	retinoic X receptor
SDS	=	sodium dodecyl sulphate
SDS-PAGE	=	SDS-polyacrylamide gel electrophoresis
SET-buffer	=	sucrose-EDTA-Tris buffer
SM	=	sphingomyelin
StAR	=	steroidogenic acute regulatory protein
START domain	=	StAR-related lipid transfer domain
SUVs	=	small unilammellar vesicles
TBS	=	Tris-buffered saline
TG	=	triglycerides
TUDC	=	tauroursodeoxycholate
VLDL	=	very low-density lipoprotein

Chapter 1

Introduction

Phospholipid transfer proteins

The first phospholipid transfer protein was discovered in 1968 by Wirtz and Zilversmit (1). They observed that *in vitro* the addition of membrane-free cytosol from rat liver resulted in a redistribution of radiolabelled lipids between mitochondria and microsomes. A water-soluble protein was identified mediating the redistribution of lipids between these membranes. In the decade thereafter it became apparent that there were a number of phospholipid transfer proteins present in mammals, plants, various yeast strains and even bacteria (2). Phospholipid transfer proteins are usually classified according to their specificity for the various head-groups of phospholipids. Currently, four classes of phospholipid transfer proteins are known: 1) phosphatidylcholine transfer protein (PC-TP), which is specific for phosphatidylcholine (PC) (3-6); 2) phosphatidylglycerol transfer protein (PG-TP) that is found to transfer specifically phosphatidylglycerol (PG) (7); 3) phosphatidyl-inositol transfer protein (PI-TP), which displays a marked preference for phosphatidylinositol (PI), but is also able to transport PC or PG (8-11); and 4) non-specific transfer protein (nsL-TP), which can transfer virtually all lipids (12, 13). The sequence homology among these phospholipid transfer proteins is very low or even non-existing. However, there are clear structural homologies among the lipid-binding sites of these proteins as well as other lipid transporting proteins, such as the steroidogenic acute regulatory protein (StAR) (14-17). Although at first described as mere transporters of phospholipids between membranes, evidence is emerging depicting a role of phospholipid transfer proteins in specific cellular processes. PI-TP is though to be involved in phosphatidylinositol signalling (18-21), activation of phosphoinositide kinases (22-24) (21), phospholipase A_2 activation (25) and vesicular transport (26-28). nsL-TP is most probably involved in the β-oxidation of branched chain and long chain fatty acids in the peroxisomes, despite its ability to transfer a wide variety of lipids (29-31). Unambiguous proof for a physiological function of PC-TP or PG-TP is still lacking.

Phosphatidylcholine transfer protein

The phosphatidylcholine transfer protein (PC-TP) was originally isolated from bovine liver cytosol in 1973 by Kamp *et al.* (3). In the years thereafter the presence of PC-TP was demonstrated in the cytosolic fraction of rat liver (4, 32), sheep lung (5), human erythrocyte haemolysate (33) and porcine liver (6) as well. In non-mammalian eukaryotes such as *Saccharomyces cerevisiae*, *Caenorhabditis elegans* or *Drosophila melanogaster*, PC-TP or a homologue could not be identified as determined by BLAST (34).

PC-TP is highly conserved among species (figure 1) (35-39). The complete deduced amino acid sequence of bovine, human, rat and murine PC-TP is more than 84% similar, whereas the amino acid residues reported to be part of the lipid-binding site accommodating the *sn*-2 acyl chain of PC are fully conserved (40-42). Although the sequences are quite similar (84-92%), there are a number of marked differences between the PC-TP isoforms (table 1). Bovine PC-TP lacks one amino acid on the C-terminus and consequently consists of 213 amino acid residues instead of 214. The isoelectric point (pI) of rat PC-TP is 8.4 (4, 32, 43), whereas the pI of the other PC-TP's is acidic as determined theoretically or by experimental procedures (44, 45). Notably, antibodies generated against rat PC-TP or mouse PC-TP do not recognise bovine PC-TP and *vice versa* (32)(A.P.M. de Brouwer, unpublished results).

```
          1
muPC-TP   MAGAACCFSD EQFREACAEL QKPALTGADW QLLVEASGIT IYRLLDQPSG LYEYKVFGVL
ratPC-TP  ---P-AH--- ---------- ---------- ---------- -------ST- ----------
huPC-TP   -EL--GS--E ---W------ -Q---A---- -----T---S ------KKT- ----------
bosPC-TP  -DPG-GA--E ---------- -R---S--A- E----TQ--S V------QT- --A-------

          61
muPC-TP   EGCSPALLTD VYMDLDYRKQ WDQYVKELYE KESDEQMVAY WEVKYPFPLS NRDYVYTRQR
ratPC-TP  -S-I-S--A- ---------K ---------- -SF-G----- ---------- ----------
huPC-TP   -D---T--A- I---S----- ---------- Q-CNGET-V- --------M- ------L---
bosPC-TP  -D-L-D--A- -----A---- ---------- --CSGET-V- -Q------M- ------V---

          121
muPC-TP   RDLDVDRRKI YVVLAQSISA PQFPEKSGVI RVKQYKQSLA IESDGKKGSR VFMYYFDNPG
ratPC-TP  ------G--- ------N--V ---------- ---------- ---------- ----------
huPC-TP   ----ME---- H-I--R-T-M --LG-R---- ---------- ---------K ----------
bosPC-TP  QE--FE-Q-V H-I----T-E ---------- ---H---R-- -Q-------K ----------

          181                               214
muPC-TP   GQIPSWLINW AAKNGVPNFL KDMVKACQNY HKKT    similarity   identity
ratPC-TP  ---------- -------S-- ---------- ----       92.1         92.1
huPC-TP   ---------- ---------- ---AR----- L---       85.8         81.0
bosPC-TP  ------V--- ---------- ---------- *---       84.0         77.4
```

Figure 1. **Sequence alignment of the deduced amino acid sequence of mammalian PC-TP's given in one-letter code.** Amino acid sequences of murine (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 grey, and amino acids with a lower similarity are shaded in light grey. The overall similarity and identity of the PC-TP isoforms to murine PC-TP are indicated.

Table 1. **Characteristics of mammalian PC-TP isoforms.** N.d. means not determined; Ab stands for anti-bodies.

PC-TP isoform	Number of amino acid residues	Mw	pI	Recognised by bovine PC-TP Ab	Recognised by rat PC-TP Ab	Recognised by mouse PC-TP Ab
Bovine	213	24,681	5.8	+	-	-
Rat	214	24,833	8.4	-	+	+
Murine	214	24,785	6.6*	-	n.d.	+
Human	214	24,843	5.6*	+	n.d.	n.d.

*pI values were calculated according to Wilkins *et al.* (45)

In the literature the presence of two disulphide bridges between Cys^{17}-Cys^{63} and Cys^{93}-Cys^{207} of bovine PC-TP is described (35). These disulphide bridges are supposed to stabilise the protein to enable PC binding and transport. Accordingly, PC-TP looses activity in the presence of dithiothreitol (DTT) (46). Normally, only secreted or cell surface proteins form disulphide bridges to stabilise their three-dimensional structure in a 'hostile' environment. In fact, disulphide bonds are rarely, if ever, formed in proteins in the cytosol because of the high cytosolic concentration of reducing agents (47). Since PC-TP is present in the cytosolic fraction and Cys^{93} is not conserved between the isoforms, we had reservations about the observation that the formation of these disulphide bridges is required for the transfer activity of PC-TP. In contrast to the previous study, we found that 0-200 mM DTT did not affect the transfer activity of native bovine PC-TP (figure 2). The presence of DTT is even indispensable to activate PC-TP from inclusion bodies (chapter 2). Furthermore, modification of cysteine residues by iodoacetamide did not cause a loss of activity (chapter 4). Therefore, we consider the reported disulphide bonds, if present in PC-TP at all, as non-essential for the stabilization and activity of PC-TP.

Mode of action

In vitro studies showed that the transfer of PC involves a one-for-one molecular exchange (48, 49). This agrees with the observation that upon purification PC-TP contains PC in a 1:1 stoichiometric complex (48, 50). At the recipient membrane PC-TP delivers its bound PC and exchanges it with PC present in that membrane; a process that is energy-independent (figure 3). Both electrostatic and hydrophobic requirements have to be met for a lipid molecule to be exchanged at the membrane interface. Minor modifications of the head group of PC affect transport considerably (50). Transfer is inhibited or abolished when the distance between the phosphorus

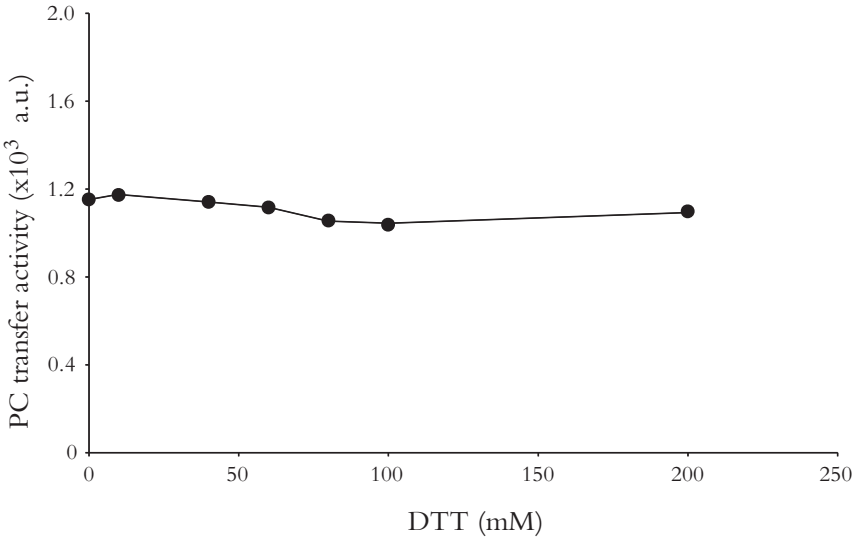

Figure 2. **Effect of DTT on the transfer of PC by PC-TP.** PC transfer activity was determined according to the continuous fluorescence assay described by van Paridon et al. (138). 0.25 µg PC-TP was tested in the presence of the various concentrations DTT. If no PC-TP was added, no transfer activity was observed.

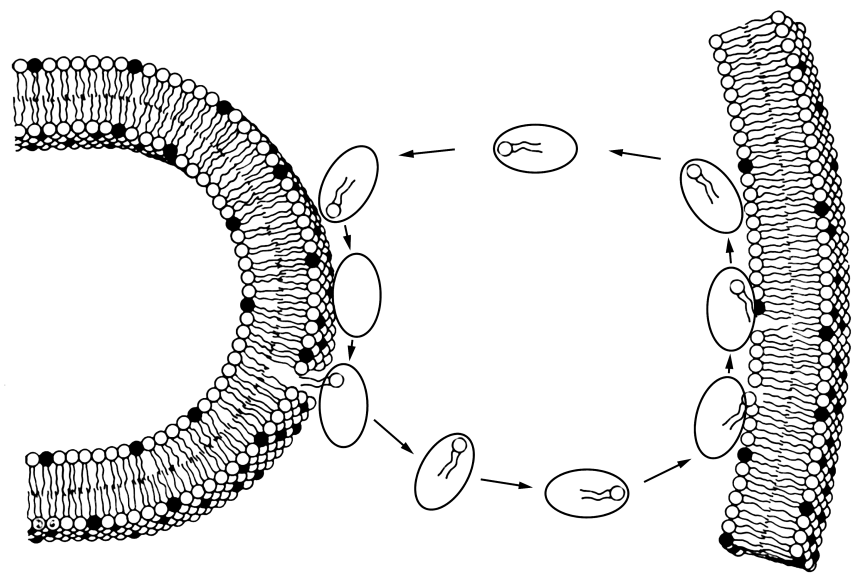

Figure 3. **Representation of the shuttling of PC between membranes by PC-TP.** For detailed explanation see text.

and nitrogen is decreased or increased and a methyl group on the quaternary nitrogen is removed or substituted by an ethyl or propyl group. Binding of PC is less affected by the composition of the acyl chains. Small modifications of the acyl chains, such as replacing the ester bond on the sn-1 position for an ether bond, do not affect PC transfer (50). On the other hand, neither 1-acyl-lysoPC (50) nor a dimeric form of PC consisting of two palmitoyl-PC molecules linked together by a disulphide bond at the methyl terminal ends of the 1-acyl chains are transported by PC-TP (51).

The transfer rate of PC-TP can be regulated by the lipid composition of the membrane. An increase in fluidity (52, 53) and a higher curvature of the membrane enhance PC transfer (54, 55). Both processes are thought to facilitate the penetration of PC-TP into the bilayer. Traffic is also affected by the presence of specific phospholipids in these membranes. Negatively charged phospholipids, such as phosphatidylserine (PS), phosphatidic acid (PA), PG and PI, increase the association of PC-TP with membranes (55, 56). When both acceptor and donor vesicles have a similar acidic phospholipid content, the transfer of PC is enhanced concomitant with an increase in negatively charged phospholipids (10, 55, 57-59). In addition, several ions are reported to affect PC transfer rates by their interactions with lipids in the membrane. Ca^{2+}, Mg^{2+} and K^{+} regulate the exchange reaction by neutralising the negative charges of the anionic phospholipids (60, 61). When membranes containing solely PC were used, cations inhibited the transfer in the order $La^{3+} > Mg^{2+} \geq Ca^{2+} > K^{+} = Na^{+}$. Inhibition was not related to ionic strength, but very likely reflects an interference of these cations with an electrostatic interaction between the exchange protein and the polar head group of PC (60).

PC-TP can cause a net transfer of PC to donor membranes and consequently be void of PC yet still active. Using electron-spin resonance and resonance-energy transfer spectroscopic techniques, it was shown that PC-TP can cause net transfer of PC to membranes lacking this phospholipid (51, 55, 62, 63). In these cases, PC-TP is thought to return to the PC containing donor membranes without any phospholipid attached. This transfer rate is markedly increased if PC is present in both membranes (51). It is doubtful if this net transfer also occurs in living cells, since PC comprises at least 30 % of all the phospholipids in cellular membranes (64).

Conformation of the lipid-binding site

PC carried by PC-TP resides in an enclosed internal cavity completely shielded from the medium (16, 65-67). Consequently, phospholipase A_2 (PLA_2), C (PLC) and D (PLD) cannot hydrolyse PC bound by PC-TP (49). This is confirmed by the structure of the steroidogenic acute regulatory protein (StAR)-related lipid transfer

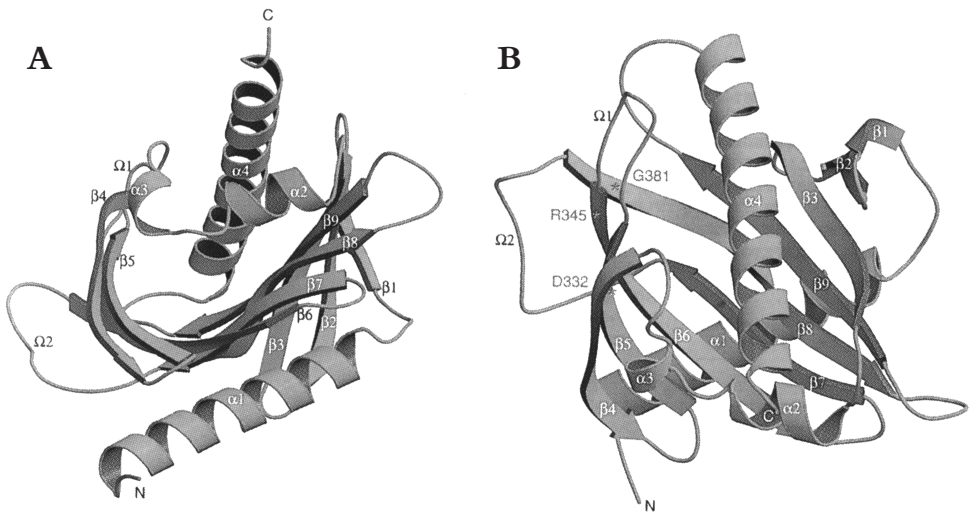

Figure 4. **Overall structure of the START domain of MLN64 as depicted by ribbon diagrams of MLN64 START (16)**. View B is related to view A by a 90° rotation about the x-axis.

(START) domain found in a number of lipid transporting proteins and for which PC-TP is representative (16). Based on the crystal structure of the START domain of human MLN64 (figure 4), it was proposed that PC-TP forms a hydrophobic tunnel in which PC is accommodated with the polar head group interacting with Arg^{78} and Asp^{82} present in a loop at the surface of the protein. This agrees with Tyr^{77} being located on the surface of the protein as demonstrated by its accessibility for iodination with lactoperoxidase (68). Furthermore, the Arg^{78} might well be the essential arginine residue identified by the membrane-permeable reagents butanedione and phenylglyoxal (69).

The two acyl chains of PC are positioned separately inside the PC-TP molecule (70). Tyr^{54} and Val^{171}-Asn^{177} are part of the peptide segments that accommodate the *sn*-2 acyl chain (40-42). These residues are present in the hydrophobic regions that form the β3- and β9-sheet (figure 5). Modification of Lys^{55} that is part of the β3 sheet by the apolar reagent phenylisothiocyanate inhibited PC transfer activity (71). The presence of vesicles greatly enhanced the inhibition, indicating that Lys^{55} is part of a peptide segment that interacts with the membrane. Probing the crystal structure of the START domain more scrupulously suggests that the *sn*-1 acyl chain can be bound by the β4, β5 and β6 hydrophobic sheets, which are 95 % similar. The β5 sheet

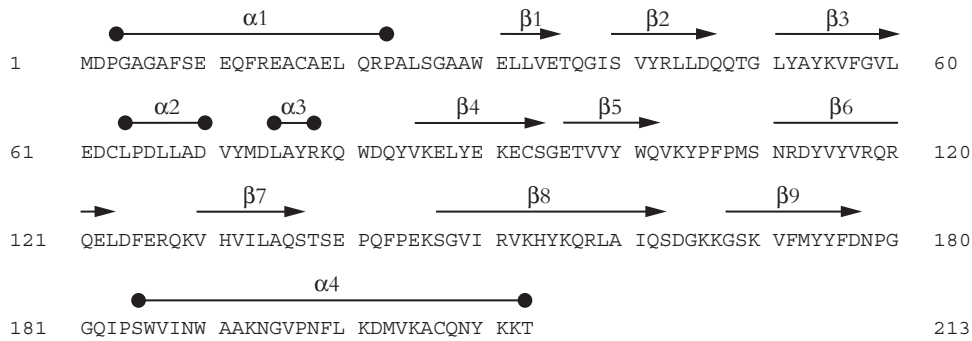

Figure 5. **Structure based alignment of bovine PC-TP (16).** Indicated are the a-helixes and b-sheets positioned according the structure of the START domain of MLN64.

contains the Val^{98}-Val^{103} hydrophobic region which is one of the most hydrophobic regions in PC-TP and thus supposedly involved in lipid binding (35).

The α4 helical loop at the N-terminus is proven to interact with the membrane interface (56). The residues Val^{196}-Thr^{214} interact with negatively charged phospholipids, which stabilise the α-helix. Subsequently, the hydrophobic region between Trp^{186}-Ala^{192} can penetrate the membrane bilayer (35, 56). Ultimately, this leads to opening of the PC-TP molecule and induces the PC exchange.

PC molecular species preference

Despite its specificity for PC, PC-TP discriminates between positional isomers of PC. Since PC-TP has separate binding sites for the *sn*-1 and *sn*-2 acyl chains of a PC molecule, it is likely that each binding site has its own specific properties. The first evidence supporting this assumption was found using radiolabelled PC species (72). The order of transfer rate of the four tested species was: C16:0/C16:0-PC < C16:0/C18:1-PC = C16:0/C18:2-PC < C18:1/C18:1-PC. It seems that on the *sn*-1 position a C18:1 acyl chain can be accommodated best, whereas on the *sn*-2 position a C18:2 or C18:1 acyl chain fits best. However, radiolabelled C14:0/C18:1 species are transferred at a higher rate than C18:1/C18:1 species, questioning the acyl chain specificity of the *sn*-1 position (73). Binding and transfer of straight acyl chains was studied using fluorescently labelled PC analogues, carrying saturated acyl chains of different lengths at either the *sn*-1 or *sn*-2 positions and a pyrene labelled acyl chain on the other position (74). Binding and transfer was maximal for a C16:0 acyl

chain on the *sn*-1 and *sn*-2 position. It has to be noted that for binding and transfer of the bulky pyrene labelled acyl chains of different lengths tested, the binding site of the *sn*-2 position is particularly suited. In nature usually unsaturated long chain fatty acids are found on the *sn*-2 position. These PC species were tested in a competition binding assay in which the binding of various PC species to PC-TP was compared to the binding of a pyrene labelled PC analogue (75). PC-TP had the highest affinity for C16:0/C20:4-PC and C16:0/C22:6-PC. The affinity for the C18:0/C18:1-PC and C18:0/C18:2 PC species was lowest. Intriguingly, the latter two PC species are most abundant in tissues expressing PC-TP such as bovine liver (table 2). There was no difference between binding PC species carrying either a C18:1 or C16:0 on the *sn*-1 position.

Table 2. **Major molecular PC species endogenously bound by bovine liver PC-TP as compared to the major PC species present in bovine liver microsomes.** PC-TP was isolated from bovine liver as described by Westerman *et al.* (136). PC was extracted from PC-TP by the method of Bligh and Dyer (139). PC species were analysed by HPLC (137).

Molecular PC species (*sn*-1, *sn*-2)	Bovine liver PC-TP	Bovine liver
	(mol% of total species)	
C16:0, C18:1	22.1 ± 8.6	12.9 ± 0.3
C16:0, C18:2	17.6 ± 2.2	10.7 ± 0.3
C16:0, C20:4	20.1 ± 1.4	6.5 ± 0.3
C18:0, C18:1	4.6 ± 1.2	25.9 ± 0.3
C18:0, C18:2	7.6 ± 2.3	31.5 ± 0.9
C18:0, C20:4	28.0 ± 2.7	12.6 ± 0.3

In summary, PC-TP has the highest affinity for the naturally occurring PC species carrying a C16:0 acyl chain on the *sn*-1 position and a polyunsaturated fatty acid (PUFA) on the *sn*-2 position of PC. In contrast, PC extracted from purified bovine liver PC-TP was described to contain only C18:0 and C18:1 acyl chains on the *sn*-1 position of PC and a PUFA on the *sn*-2 position (76). However, verification of these results by four other bovine liver PC-TP isolates revealed that on the *sn*-1 position a C16:0 chain was preferred (table 2). The former results were possibly caused by an artefact during isolation.

Table 3. **Tissue distribution of PC-TP in adults (77,36,38).** The relative expression levels are indicated by – (not detectable) to +++++ (highest expression). N.d. means not determined.

Tissue	Relative expression		
	Rat	Mouse	Human
Liver	+++++	+++++	+++++
Kidney	+	++++	++
Testis	n.d.	+++++	+++
Lung	+/-	++	++
Spleen	+	++	+/-
Intestinal mucosa	++	n.d.	n.d.
Small intestine	n.d.	+/-	++
Colon	n.d.	+	+
Uterus	n.d.	++	n.d.
Pancreas	n.d.	n.d.	+
Stomach	n.d.	+	n.d.
Prostate	n.d.	n.d.	+
Leukocyte	n.d.	n.d.	+
Placenta	n.d.	n.d.	+
Ovary	n.d.	n.d.	+/-
Adrenal glands	+/-	n.d.	n.d.
Muscle	n.d.	+/-	-
Heart	-	+/-	+
Brain	-	-	+
Thymus	n.d.	-	-

Cellular localization and tissue distribution

PC-TP is not expressed equally in the various organs and tissues. Although in general the tissue distribution among mammals is similar, small differences could be detected (table 3). Of rat PC-TP the highest levels are measured in liver and intestinal mucosa. Lower values were found in kidney, spleen, lung, whereas heart and brain contained hardly any PC-TP (77). Mouse PC-TP is expressed highly in liver, testis and kidney. Medium expression levels are found in lung, heart, spleen, muscle and intestine, while almost no PC-TP is present in brain and thymus (36). Highest human PC-TP levels are present in liver, placenta, testis, kidney and heart. Only the thymus lacked evidence of PC-TP expression and low levels were observed in brain and lung. All other tissues examined contained considerable PC-TP mRNA levels (38). PC-TP RNA was present in embryos at all stages of development as early as the

embryonic stem cell. Towards the end of embryonic development, just before term and after term, PC-TP is expressed maximally in the liver of mice (36). This substantially exceeds the expression levels found in the liver of adult animals. Concurrently, PC-TP levels are maximal 2 days before term in foetal lung of rats (77).

In general, PC-TP is expressed in tissues exhibiting a high lipid turnover such as liver and kidney. This agrees with the low expression levels in organs such as thymus and brain. This also explains the high PC-TP expression levels just before term. At that time the organism starts actively metabolising lipids.

Promotor sequence

Detailed analysis of the promoter sequence of rat *Pc-tp* gene revealed a number of binding sites for transcription factors (figure 6)(78). These transcription factors can roughly be divided into three functional groups: lipid transport/metabolism (PPAR/RXR and C/EBPβ), cell differentiation/proliferation (C/EBPβ, NF-kappa B, HNF-4, HFH-2 and Sp1) and inflammation/immune responses (C/EBPβ and NF-kappa B) (79, 80) (81, 82). The putative human promotor sequence contains the PPAR/RXR, C/EBPβ and Sp1 binding sites as well as shown by TFSEARCH version 1.3 (83).

Peroxisome proliferator-activated receptors (PPARs) are lipid-activated transcription factors that belong to the nuclear hormone receptor superfamily. They play a key role in lipid metabolism and homeostasis (84, 85). PPAR target genes encode enzymes involved in peroxisomal and mitochondrial β-oxidation and ketone body synthesis, as well as fatty acid binding proteins, apolipoproteins and lipoprotein lipase. Three PPAR subtypes have been characterised: α, β and γ. Upon dimerization with 9-*cis*-retinoic acid receptor (RXR), PPARs typically bind to DNA elements consisting of a degenerate direct repeat of the recognition motif TGACCT spaced by one nucleotide (DR1 element) enhancing gene transcription. Of the PPARs, PPARα is the most likely candidate to regulate PC-TP expression, since its tissue distribution is similar to that of PC-TP. In adult rat, PPARα is mostly detected in liver, kidney, heart, brown adipose tissue and the intestine (86-88).

The CCAAT/enhancer binding protein β (C/EBPβ) regulates genes involved in inflammatory responses, lipid storage and gluconeogenic pathways, such as tumor necrosis factor (TNF), interleukin 8 (IL-8) and phosphoenolpyruvate carboxykinase (80). Constitutive expression of C/EBPβ is highest in liver, intestine, lung and adipose tissue (89-91). Considerable mRNA levels are also found in kidney, heart and spleen (90).

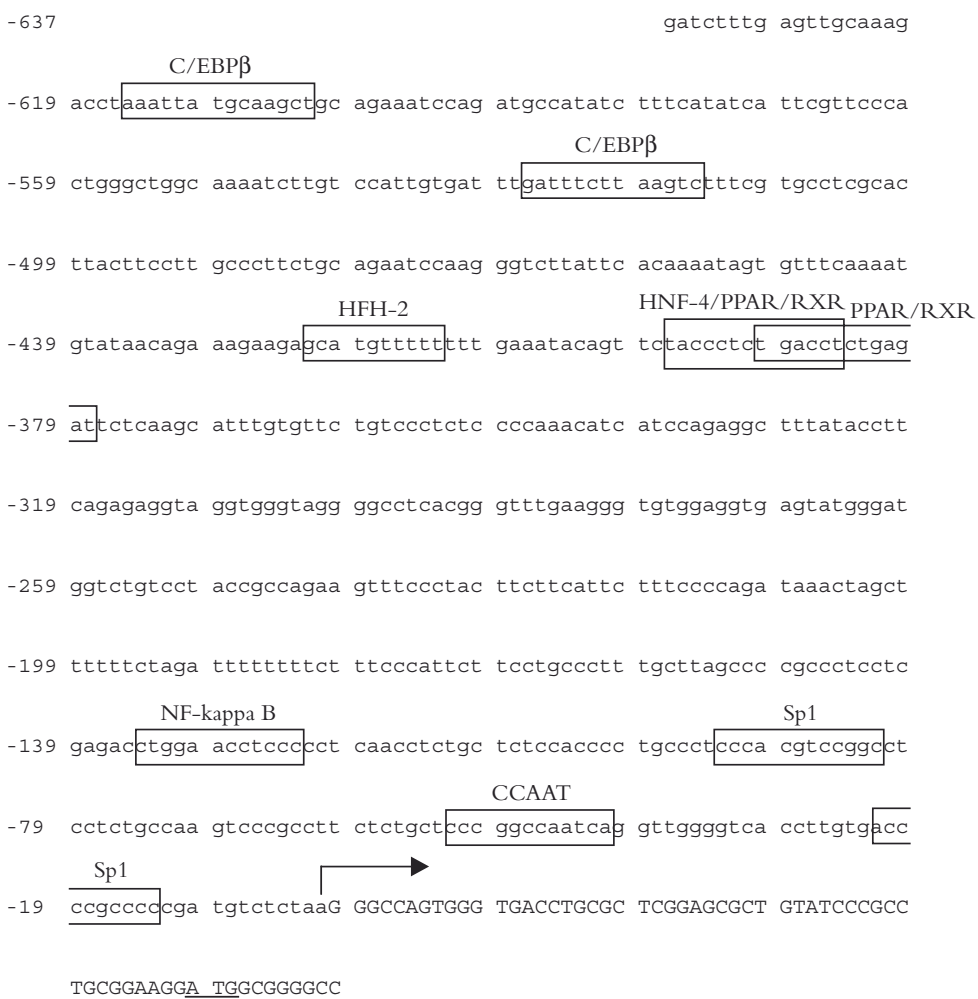

Figure 6. **Nucleotide sequence of the 5'-flanking region of the rat *Pc-tp* gene.** Boxes indicate consensus sequences for binding of common transcription factors. The putative transcription initiation site is indicated by an arrow and the triplet encoding the first methionine residue of the protein is underlined (reproduced from (78)).

NF-κB plays a central role in inflammation through its ability to induce transcription of proinflammatory genes (82). Synthesis of cytokines, such as TNF-α, IL-6 and IL-8 is mediated by NF-κB, as is the expression of cyclooxygenase 2 (Cox-2). In addition, since NF-κB can induce FasL and caspase-8 expression, it is also

implicated in apoptosis. NF-κB is present in a great variety of cells capable of eliciting inflammation, such as monocytes, lymphocytes, vascular endothelial cells, airway/colon epithelial cells, hepatocytes and nephritic glomeruli (92-96).

Hepatocyte nuclear factor-4 (HNF-4) is one of the liver enriched transcription factor proteins (97). The HNF-4 gene is proposed to be a tumour suppressor gene that plays an important role in differentiation and anti-proliferation during development. HNF-4 is primarily expressed in liver, kidney and intestine in the adult (98), and activates a diverse set of liver genes such as transthyretin and α1- antitrypsin in early liver development (99). In analogy, the HNF-3/forkhead (FKH) homologue-2 (HFH-2) is also thought to play a role during development (79). HFH-2 is expressed in liver, kidney, lung, small intestine, heart and brain (100).

The Sp1 transcription factor binding site is a frequently occurring DNA-element present in many promoters and enhancers (101). Sp1 is implicated in the activation of house-keeping, tissue-specific and cell cycle-regulated genes and appears to be essential for differentiated cells after day 10 of development as determined by disruption of the mouse *Sp1* gene.

In general, the distribution of the transcription factors that can enhance PC-TP expression coincides with the expression pattern displayed by PC-TP. In adult animals, high expression levels are found in liver, kidney, intestine and lung. The presence of binding sites for transcription factors involved in development also explains the expression of PC-TP as early as the embryonic stem cell (36).

Physiological function

Based on PC specificity, PC species preference, tissue distribution and promotor sequence, a number of functions for PC-TP can be postulated. First of all, PC-TP could play a role in bile formation. PC is by far the predominant phospholipid class found in bile, accounting for more than 95% of secreted phospholipids (102, 103). Intriguingly, bile PC is enriched in C16:0/C18:1-PC and C16:0/C18:2-PC whereas PC species carrying a C18:0 acyl chain at the *sn*-1 position are hardly found (104-107). Secretion of PC is regulated by the mouse MDR-2 P-glycoprotein (108). This P-glycoprotein is thought to act as a PC translocator, flipping PC from the inner to the outer leaflet (108-111). PC-TP may contribute to this process by transporting PC from its site of synthesis, the endoplasmic reticulum, to the inner leaflet of the canalicular membrane (108). This role for PC-TP in bile formation is supported by evidence resulting from multivariate analysis used to examine the relationship between PC molecular species present in the liver and the odds of secretion for individual PC species secreted into bile (112). It was calculated that there is a linear

relationship between the odds of secretion and the affinity for PC-TP for naturally occurring PC species. This suggests that the likelihood of a PC being secreted into bile is closely related to its binding affinity for PC-TP. In addition, PC-TP-mediated transfer of PC between membranes is stimulated by submicellar concentrations of the common bile salts (113). Furthermore, PC-TP levels in the human liver correlate with amount of MDR-3 P-glycoprotein (the human orthologue of mouse MDR-2 P-glycoprotein) and biliary phospholipid secretion (114). The prominent presence of PC-TP in developing and adult mouse liver is compatible with its proposed role in bile formation as well (36).

Parallel to PC-TP, human apolipoprotein genes are transcriptionally regulated by PPAR, C/EBP, HNF-4 and Sp1 (78, 115). Lipoproteins are composed of triglycerides, cholesterol and phospholipids consisting for a substantial amount of PC (116). Furthermore, by perfusion of isolated rat livers with or without the bile salt taurocholate, it was proposed that the PC species in bile and high-density-lipoprotein (HDL) originate from the same hepatic pool or by the same mechanism (117). In analogy to bile, PC-TP could regulate the formation of lipoprotein particles.

Since PC-TP levels in foetal lung of rats are maximal just before term (77), a role for PC-TP in the secretion of lung surfactant can be suggested. Pulmonary surfactant consists for 90% of phospholipids of which PC is the major component (118-120). Surfactant is produced by the alveolar type II cells, which are highly enriched in phospholipid transfer activity as compared to the whole lung (121). If PC-TP is chiefly expressed in these cells, this could account for the medium expression levels in adult lung. On the other hand, surfactant PC consists for 60% of C16:0/C16:0-PC species (118-120), which are not preferred by PC-TP as compared to other PC molecular species (75).

PC-TP is highly expressed in tissues displaying a high lipid turnover. This could indicate a role for PC-TP during PC synthesis. PC can be synthesised either by the Kennedy pathway (or CDP-choline pathway) or by methylation of phosphatidylethanolamine (PE) (122, 123). The final step of the Kennedy pathway involves the reaction between CDP-choline and diacylglycerol (DAG) to yield PC and CMP. This step is catalysed by CDP-choline:DAG choline transferase. PC-TP is able *in vitro* to stimulate the cholinetransferase activity (124). Although the Kennedy pathway is the major source of *de novo* synthesised PC, in liver a substantial amount of PC can be generated by the PE methyl transferase (PEMT) pathway. The liver specific PEMT methylates PE generating PC. Although PC-TP is more ubiquitously expressed than just in the liver, it could play a role in regulating this enzyme, especially since PC synthesised from PE is comprised of significantly more long chain

polyunsaturated molecular species (125).

PC-TP could also be involved in hydrolysis of PC by phospholipases. PC-TP has the highest affinity for PC species containing PUFAs which can act as precursors in the prostaglandin/leukotriene biosynthesis (126). This would explain the presence of binding sites for transcription factors involved in the regulation of inflammation. PC-TP could be involved in the delivery of these species to PLA_2 releasing the PUFAs. According to this model, PC-TP plays a similar role as is proposed for PI-TPα (18, 20-22, 25). In addition, $PLA_{1/2}$ plays an important role during deacylation-reacylation, thereby remodelling PC. *De novo* synthesis of PC yields primarily four molecular species: C16:0/C18:1-PC, C16:0/C18:2-PC, C16:0/C22:6-PC and C18:1/C18:2-PC. C18:0 and C20:4 fatty acids are incorporated by the deacylation-reacylation reaction (127, 128). Remodelling is also reported to repair oxidised fatty acids of which especially PUFAs are prone to oxidation (129).

PC-TP could offer specific PC species to other phospholipases, such as the PC-specific phospholipase C (PC-PLC) or D (PC-PLD) as well. Along these lines, it is of interest to note that the activity of PC-PLD is markedly stimulated by unsaturated but not by saturated fatty acids (130). However, in general it is believed that hydrolysis of PC leads to saturated DAG and PA species (131), whereas PC-TP clearly prefers unsaturated PC species.

Finally, PC-TP could also deliver a PC molecule to sphingomyelin (SM) synthase, an enzyme which catalyses the conversion of PC and ceramide to SM and DAG (132-134). *In vitro*, PC-TP is shown to enhance SM synthesis (135).

Scope of this thesis

Despite extensive research concerning the biochemical, biophysical and structural properties of PC-TP, *in vivo* proof in support of a physiological function is lacking. In this thesis the key objective is to resolve the physiological function of PC-TP.

In chapter 2, the expression of bovine PC-TP in *Escherichia coli* is described. Recombinant PC-TP can be a useful tool to study structural and cell biological aspects of PC-TP. In chapter 3, this recombinant PC-TP as well as native PC-TP were used to determine the PC species affinity in the presence of microsomes and PC vesicles. This PC species affinity is supposed to be of major importance for the functioning of PC-TP, since specific PC species are involved in a number of cellular processes such as bile formation or hydrolysis by phospholipases. These processes occur at specialised sites in the cell. Therefore, we studied the subcellular localization of PC-TP in endothelial cells in chapter 4. We also attempted to cause a redistribution of PC-TP, affecting cellular processes. Therefore we tested a number of

compounds regulating PC metabolism. The peroxisome proliferators clofibrate, phytanic acid and arachidonic acid caused a relocation of PC-TP to the mitochondria. In chapter 5, the role of PC-TP in PC synthesis or breakdown in equilibrium and under conditions altering the location of PC-TP was assessed. In chapter 6, *Pc-tp* null and wild type mice were studied. Attention was focussed on a role for PC-TP in bile formation, lung surfactant production and the facilitation of enzymatic reactions involving PC synthesis or degradation. Subsequently, these mice were challenged by clofibrate and phytol containing diets (chapter 7). Evaluation of the data indicates that PC-TP is involved in regulating lipid homeostasis as is discussed in the final chapter 8.

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Chapter 2

Expression of bovine phosphatidylcholine transfer protein in *Escherichia coli*. Purification, characterization, and refolding from inclusion bodies

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Abstract

Bovine liver phosphatidylcholine transfer protein (PC-TP) has been expressed in *Escherichia coli* and purified to homogeneity from the cytosol fraction at a yield of 0.45 mg PC-TP per 10 mg total cytosolic protein. In addition, active PC-TP was obtained from inclusion bodies. An essential factor in the activation of PC-TP was phosphatidylcholine (PC) present in the folding buffer. PC-TP from the cytosol contains phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) with a preference for the di-monounsaturated species over the saturated species as determined by fast atom bombardment mass spectrometry (FAB-MS). PC-TP is structurally homologous to the lipid-binding domain of the steroidogenic acute regulatory protein. Replacement of Lys^{55} present in one of the β-strands forming the lipid-binding site, with an isoleucine residue yielded an inactive protein. This suggests that Lys^{55} is involved in the binding of the PC molecule.

Introduction

Phosphatidylcholine transfer protein (PC-TP) catalyses *in vitro* specifically the intermembrane exchange and net transfer of phosphatidylcholine (PC) (1, 2). Upon purification from bovine liver cytosol, PC-TP contains PC in a 1:1 stoichiometric complex (1, 3). Recently, it was reported that PC-TP is representative for the StAR-related lipid transfer (START) domain occurring in a great variety of proteins involved in lipid metabolism, signal transduction and transcriptional regulation (4, 5). Based on the crystal structure of the START domain of human MLN64, it was proposed that PC-TP has a hydrophobic tunnel in which PC is accommodated with the polar head group interacting with Arg^{78} and Asp^{82} present in a loop at the surface of the protein. The Arg^{78} might well be the essential arginine residue identified by the reagents butanedione and phenylglyoxal (6). The hydrophobic tunnel consist of several parallel β-sheets containing Tyr^{54} and Val^{171}-Asn^{177} which were previously reported to be part of the peptide segments that accommodate the *sn*-2 acyl chain (7-9).

Upon arrival at the membrane the α4 helical loop at the N-terminus of PC-TP is proven to interact with the interface (10). The residues Val^{196}-Thr^{214} interact with negatively charged phospholipids, which stabilise the α-helix. Subsequently, the hydrophobic region between Trp^{186}-Ala^{192} can penetrate the membrane bilayer (10, 11). Ultimately, this leads to opening of the PC-TP molecule, exposing the lipid-

binding site to the membrane interface. Notably, modification of Lys^{55} right next to the tyrosine residue in the lipid-binding site by the apolar reagent phenylisothiocyanate inhibited PC transfer activity (12). The presence of vesicles greatly enhanced the inhibition, indicating that Lys^{55} is indeed part of a peptide segment that interacts with the membrane.

To study the exchange mechanism of PC-TP at the membrane interface in depth, a simple and effective expression system is needed to provide adequate amounts of pure protein. Hence, expression of proteins in *E. coli* is preferred. So far, the main source of PC-TP has been bovine liver cytosol (13). Recently, rat PC-TP was obtained by baculovirus-mediated expression (14). The latter authors reported that PC-TP could not be expressed in *E. coli* supposedly due to the absence of PC in this organism. Yet, these same authors succeeded in obtaining substantial amounts of human PC-TP from *E. coli*, though after resigning the open reading frame according to *E. coli* preferred codon usage (10).

In this study we have successfully expressed bovine PC-TP in *Escherichia coli*. In addition, we report that PC-TP can be activated from inclusion bodies using PC. No remodelling was needed to obtain similar amounts of recombinant bovine PC-TP as compared to recombinant human PC-TP. Furthermore, using this expression system we have also assessed the role of Lys^{55} in PC transfer activity.

Experimental procedures

Materials

Escherichia coli strain BL21(DE3) was obtained from Dr. J.H. Veerkamp (Department of Biochemistry, University of Nijmegen, The Netherlands). *E. coli* strain DH5α was purchased from the Phabagen collection (Utrecht, The Netherlands). The pET15b vector was purchased from Novagen (Madison, USA). The pBluescript $SK^{+/-}$ vector and the Quickchange site-directed mutagenesis kit were obtained from Stratagene (La Jolla, USA). Primers were synthesized by Eurogentec (Seraing, Belgium). PD-10 columns, Q-Sepharose, and Sephadex S-100 column material were purchased from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Ni^{2+}-High Bond matrix was obtained from Invitrogen (San Diego, USA). 30 and 100 kDa molecular weight cut-off filters were purchased at Amicon (Beverly, USA). Silica gel 60 plates (HP-TLC plates) were obtained from E. Merck (Darmstadt, Germany). The allsphere silica column (100 x 6.4 mm; 3 μm particles) was purchased from Alltech (Breda, The Netherlands). Egg yolk PC,

trinitrophenylphosphatidylethanolamine and phosphatidic acid (PA) prepared from egg yolk PC, 5-bromo-4-chloro-3-indoyl-phosphate *p*-toluidine salt (BCIP), *p*-nitro blue tetrazolium chloride (NBT), Protein A Sepharose CL-4B, Coomassie Brilliant Blue R-250, goat-anti-rabbit IgG conjugated with alkaline phosphatase (GAR-AP) and thrombin were purchased from Sigma (St. Louis, USA). 1-Palmitoyl-, 2-pyrenyl-decanoyl-PC was synthesised according established procedures and was a kind gift of Dr. P.J. Somerharju (University of Helsinki, Finland). Isopropyl-β-D-thiogalactopyranoside (IPTG) was obtained from Promega (Madison, USA). Non fat dry milk was purchased from Nutricia (Zoetermeer, The Netherlands).

Overexpression of His-tag PC-TP

cDNA containing the complete coding sequence of bovine liver PC-TP was isolated from a λMAX bovine liver cDNA library. The cDNA encoding PC-TP was cloned into the *Eco*RI site of pBluescript SK^+. Subsequently, the pBluescript-PC-TP was digested with *Bam*HI and the ensuing insert ligated into the *Bam*HI restriction site of the pET15b vector. A construct with the insert in the sense orientation was selected and is denoted as pET15b-PC-TP. *E. coli* strain BL21(DE3) was transformed with this construct. A 100 ml culture, grown overnight in Luria-Bertani (LB) medium containing 100 μg/ml ampicillin was used to inoculate 10 l of 2xLB medium containing 44 g KH_2PO_4, 104 g $K_2HPO_5 \bullet 3H_2O$, 5 g NH_4Cl, 500 mg $MgSO_4 \bullet 7H_2O$, 70 mg $(NH_4)2SO_4$-$FeSO_4 \bullet 6H_2O$, 2 g glucose, 100 μg thiamine and 1.5 g ampicillin, which was brought to 37°C prior to inoculation. Bacteria were grown at 37°C in a 10 l Microferm Fermentor (New Brunswick Scientific CO., Brunswick, N.J.) under constant aeration. At an OD_{600} of 1.7 the culture was induced with 0.5 mM IPTG and grown for an additional 3 h in the presence of 100 μg/ml ampicillin additionally added (the final OD_{600} was approximately 12). All subsequent manipulations were performed at 4°C.

Bacteria were harvested by centrifugation at 5,000 x *g* for 25 min in a Beckman J6-HC centrifuge. The pellet was washed once with 10 mM Tris-HCl pH 7.4, 500 mM NaCl, 10 mM β-mercaptoethanol (β-ME) and then resuspended in 400 ml of the same buffer. The resuspended cells were sonicated in portions of 30 ml for 10 x 30 sec at 80 W output with a macrotip of a Sonifier B12 (Branson Sonic Power Company, Connecticut, USA). The homogenate was centrifuged at 30,000 x *g* for 30 min in an ultracentrifuge (Beckman Spinco L2 65B). The supernatant was collected and extensively dialysed against 20 mM Tris-HCl pH 7.4, 10 mM βME. The pellet containing the inclusion bodies was resuspended in 100 ml wash buffer consisting of 50 mM Hepes pH 7.6, 50 mM KCl, 1 M Urea, 1 mM EDTA, 1 mM dithiothreitol

(DTT), 1% (v/v) Triton X-114, 100 mM L-Valine and stored at –80°C.

Purification of His-tag PC-TP

Step 1. The dialysate was applied to a Q-Sepharose column (2.2 l) and eluted with 20 mM Tris-HCl pH 7.4, 10 mM βME (1.5 l) followed by a linear gradient (2 .0 l) of 0-1.0 M NaCl in the same buffer (flow rate 5 ml/min; 10 ml/fraction). The fractions were assayed for PC-transfer activity according to van Paridon *et al.* (15). The active fractions were combined and concentrated to 50 ml using a 30 kDa molecular weight cut-off filter.

Step 2. The active pool was applied to a Sephadex S-100 column (2.0 l) and eluted with 20 mM sodium phosphate pH 7.8, 500 mM NaCl (flow rate 5 ml/min; 5 ml/fraction). The active fractions were combined and concentrated to 50 ml as described above.

Step 3. Upon addition of 500 mM imidazol to a final concentration of 10 mM, aliquots of 25 ml were applied to a 20 ml Ni^{2+}-High Bond column. Active protein was eluted with a linear gradient of 10-500 mM imidazol in 20 mM sodiumphosphate pH 7.8, 500 mM NaCl (flow rate 0.5 ml/min; 5 ml/fraction). After each step, the purity of the protein was estimated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by laser scanning densitometry. Purified PC-TP was stored in 50% glycerol (v/v) at –20°C.

Refolding of PC-TP from inclusion bodies

Resuspended inclusion bodies were sonicated in portions of 25 ml for 6 x 40 sec at 65 W output with a macrotip of a Sonifier B12. The sonicated suspension was centrifuged at 20,000 x *g* for 30 min at 4°C. The supernatant was discarded and the pellet resuspended in 200 ml wash buffer. This washing step was repeated until the supernatant was colourless. Subsequently, the pellet was dissolved in 40 ml denaturing buffer consisting of 100 mM Tris-HCl pH 9.0, 8 M Urea, 100 mM DTT, 2 mM EDTA, 1% (v/v) Triton X-114, 250 mM L-Valine. This suspension was stirred for 2 h at room temperature until the aggregates were dissolved. The solution was centrifuged at 20,000 x *g* for 2 h at 4°C. The supernatant was stored at –20°C until further use. Unfolded denatured protein was added slowly under continuous stirring to 10 ml folding buffer consisting of 110 mM Tris-HCl pH 8.7, 100 mM NaCl, 1.2 M Urea, 12.5 mM DTT, 0.2 mM EDTA, 0.1% (v/v) Triton X-114, 250 mM L-Valine. Subsequently, egg PC/PA (90/10 mol%) in ethanol (20 mM) was added to the folding buffer. PC-TP was folded overnight at 4°C under gentle stirring and purified in one step using a Ni^{2+}-High Bond column as described before.

Removal of the His-tag from recombinant PC-TP

Expression of pET15b-PC-TP yields PC-TP fused to an N-terminal peptide containing 6 histidine residues. These residues can be removed by thrombin treatment leaving 6 additional amino acid residues (G-S-H-M-L-E) attached to the N-terminus. To achieve complete cleavage, thrombin (5 units) was added to 1 mg purified His-tag PC-TP in the presence of 0.2 mM DTT and 1 mM EDTA. After incubation for 72 h at 4°C under gentle rotation, the reaction product was analysed on SDS-PAGE and assayed for PC transfer activity as described above.

Site-directed mutagenesis

PC-TP cDNA was cloned into the pBluescript SK^- vector. Using the Quickchange site-directed mutagenesis method Lys^{55} was replaced with isoleucine. The following primers were used: 5'-g act gga ttg tac gct tat **ata** gtc ttt ggt gtt ctg-3' (sense) and 5'-c cag aac acc aaa gac **tat** ata agc gta caa tcc agt-3' (antisense). The bold printed nucleotides encode the mutated amino acid (Lys^{55} to Ile). The underlined nucleotides remove a *Mlu*I restriction site without changing the amino acid composition. Incorporation of the changed nucleotides into the construct was checked by restriction enzyme analysis and DNA sequencing. Mutated PC-TP was cloned into the pET15b expression vector and its activity monitored by the PC-transfer assay as described above.

Gel electrophoresis and Western blotting

Recombinant and native PC-TP were analysed by SDS-PAGE in 12% acrylamide and 0.37% bis-acrylamide (16). Proteins were visualized by Coomassie Brilliant Blue R-250 staining or transferred to a nitrocellulose membrane (Schleicher & Schuell BA 85) by semi-dry Western blotting in a Multiphor II Nova Blot electrophoretic transfer unit (Pharmacia, Uppsala, Sweden) at 1 mA/cm^2 for 1 h at room temperature. The non-specific binding sites of the nitrocellulose membrane were blocked by incubating the membrane for 1 h in 2% non fat dry milk (w/v) in 20 mM Tris-HCl pH 7.5, 0.5 M NaCl (TBS) at 37°C. Subsequently, the blot was incubated for 1 h at 37°C with the affinity purified antibody Ab270 raised against native bovine PC-TP (affinity purified by Protein A Sepharose CL-4B) diluted 1:20 in TBS containing 0.2% non fat dry milk (w/v). The blot was washed with TBS-Tween 20 (0.05% v/v; TBS-T) (3 x 10 min) and incubated for 1 h with GAR-AP diluted 1:5000 in TBS. The blot was washed again with TBS-T (3 x 10 min). The immunoreactive proteins were visualized by incubating the blot in 10 ml 0.1 M $NaHCO_3$ pH 9.8, 10 mM $MgCl_2 \cdot 6H_2O$ containing 0.3 mg/ml BCIP and 0.15

mg/ml NBT as the colour development substrate.

N-terminal amino acid sequencing

N-terminal amino acid sequence determination was carried out on the Protein Sequencer model 476A (Perkin Elmer Biosystems) by the Sequence Centre Utrecht (Utrecht University, The Netherlands)

Circular dichroism

The storage buffer of recombinant and native PC-TP was exchanged for a 20 mM Tris-HCl pH 7.4, 100 mM NaCl buffer using a PD10 column. Circular dichroism spectra were recorded at room temperature on a JASCO J-600 spectropolarimeter (Japan Spectroscopic Co. LTD., Hachioji City, Tokyo, Japan).

Analysis of lipids

Lipids were extracted from recombinant PC-TP by the method of Bligh and Dyer (17). The phospholipids bound to PC-TP before and after exchange were separated by high performance thin layer chromatography (HP-TLC) using chloroform/methanol/water 65/25/4 (v/v/v) as mobile phase (18). The phosphorus content of the individual spots was determined according to Rouser *et al.* (19). The phospholipid molecular species were quantified by negative and positive ion fast bombardment mass spectrometry (20).

Results

Expression and purification of His-tag PC-TP

Expression of the pET15b-PC-TP construct in *Escherichia coli* strain BL21(DE3) yielded PC-TP present both in the cytosol and in inclusion bodies. His-tag PC-TP from the cytosol was purified to homogeneity using a three-step procedure (table 1). Upon induction PC-TP was not clearly visible in the total cytosol fraction (figure1A; lane1). After application of this fraction to a Q-Sepharose column (step 1), PC-TP eluted in the run-through resulting in a two-fold purification. A major purification was achieved by running the active fraction on a Sephadex S-100 column (step 2). By laser scanning densitometry it was estimated that 14 % of the active pool was PC-TP (figure 1A; lane 3). Application of this pool to a Ni^{2+}-High Bond matrix column (step 3) yielded a homogeneous PC-TP fraction (figure 1B; lane 1). After removal of the His-tag by thrombin, a single protein was obtained with an estimated M_r of 28

Table 1.
Purification scheme leading to homogenous His-tag PC-TP from the cytosol of *Escherichia coli*.

Step	Total protein (mg)	Purity (%)	Recovery (%)
30,000 x *g* supernatant	22,222	0.9	100
(1) Q-Sepharose	8,105	1.9	77
(2) Sephadex S-100	778	14	55
(3) Ni^{2+}-High Bond	97	100	49

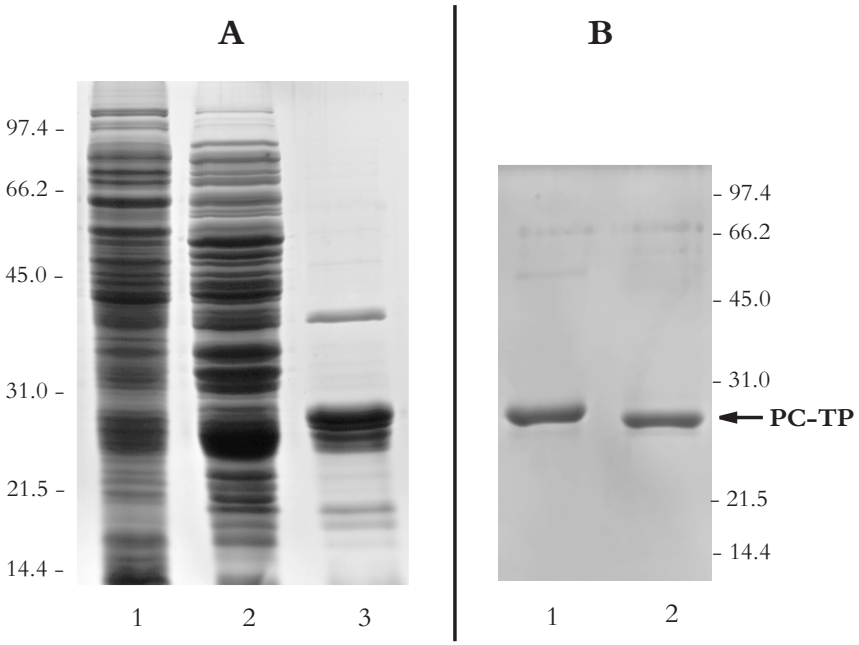

Figure 1. **Purification of recombinant PC-TP as determined by SDS-PAGE analysis.** Panel A. Lane 1: 30,000 x *g* bacterial supernatant; lane 2: PC-TP pool after Q-Sepharose anion exchange chromatography; lane 3: PC-TP pool after Sephadex S-100 molecular sieve chromatography. Panel B. Lane 1: recombinant PC-TP before His-tag removal by thrombin; lane 2: recombinant PC-TP after removal of the His-tag.

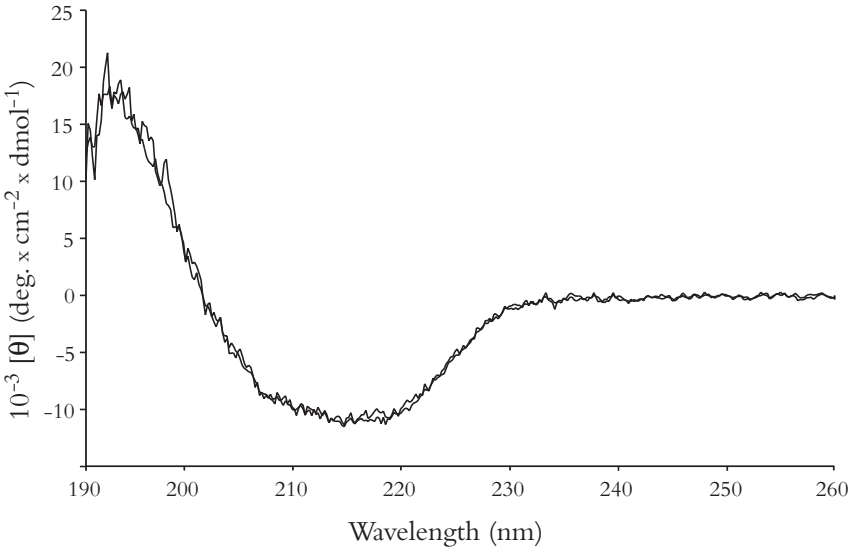

Figure 2. **Circular dichroism spectra of 2 μM native and recombinant PC-TP in 20 mM Tris-HCl pH 7.4, 100 mM NaCl**.

kDa, which is in agreement with that of pure native protein (figure 1B; lane 2). Using the molecular extinction coefficient of 60,365 $M^{-1}cm^{-1}$ (11), the final yield was estimated to be 97 mg of protein (49% recovery). By Edman degradation it was established that the first six amino acid residues of the N-terminus consisted of Gly-Ser-His-Met-Leu-Glu confirming that the isolated protein originated from the pET15b-PC-TP construct. The activity of the purified recombinant PC-TP was comparable to that of native PC-TP. In agreement with this, the circular dichroism spectra were identical (figure 2). Evaluation of secondary structure of proteins from UV circular dichroism using the unsupervised learning neural network k2d predicts the content of α-helix and β-sheet to be 26% and 43%, respectively (21, 22). Although this prediction method is based on globular proteins and therefore not fully accurate, this comes close to the estimated value of 29% α-helix and 41% β-strand based on the crystal structure of the START domain of human MLN64 (5). In addition, the PC species preference and subcellular localization of recombinant PC-TP were similar to those of native PC-TP (chapter 3; figure 1 and chapter 4; figure 2)

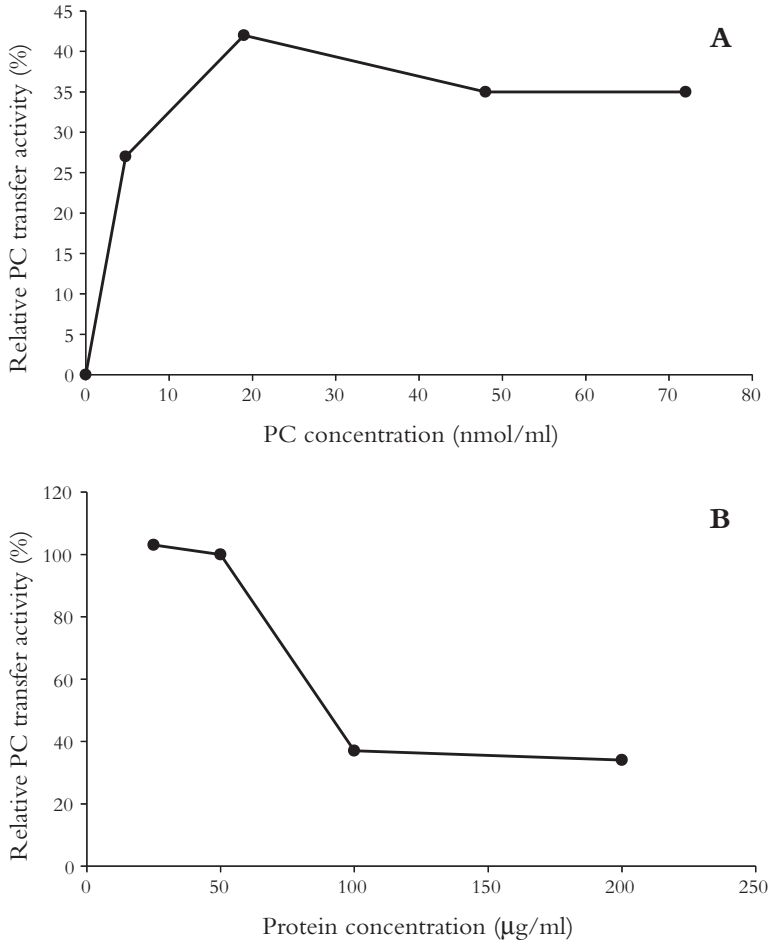

Figure 3. **The effect of different phosphatidylcholine and protein concentrations on the folding of PC-TP from inclusion bodies as determined by PC transfer activity.** Panel A: effect of various PC concentrations. Panel B: effect of various protein concentrations. The activity of native bovine PC-TP is set at 100 %.

Folding from inclusion bodies

An estimated 90% of all PC-TP expressed in *E. coli* accumulates in inclusion bodies. To maximize the yield of protein, conditions have been established to obtain active protein from these aggregates. As a first step the aggregated proteins were dissolved in denaturing buffer containing Triton X-114, L-valine and EDTA to stabilize the protein. After denaturation the protein was folded under conditions

which allow the protein to adopt the proper conformation. This was achieved by diluting the denatured protein in the folding buffer. These denaturation and folding buffers were based on the ones successfully applied in the refolding of recombinant phosphatidylinositol transfer protein (23). As shown in figure 3A, PC-TP could only be activated when PC was present in the folding buffer. PC was added in combination with PA so as to facilitate the uptake of PC by PC-TP (24). Optimal activation of PC-TP (100 μg/ml) was achieved at concentrations between 20 and 80 μM PC. To attain an activity comparable to that of native PC-TP, the protein concentration during folding must be equal to or lower than 50 μg/ml (figure 3B). At higher concentrations folding appears to be less efficient as the recovery of activity was substantially lower. Purification of refolded PC-TP was achieved in one step using immobilized metal ion chromatography (figure 4). Approximately 1.5 g of protein can be obtained from of a 10 l culture.

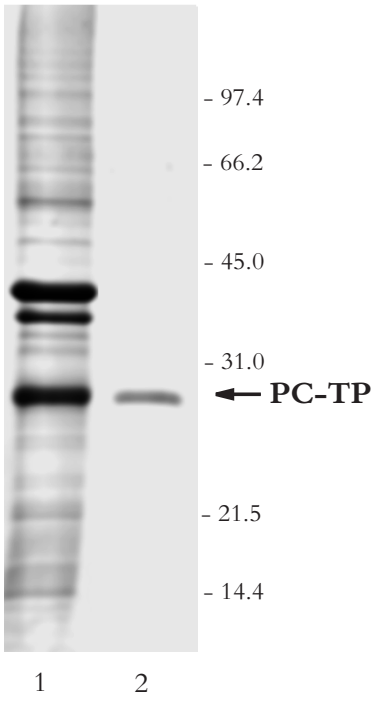

Figure 4. **SDS PAGE analysis of recombinant PC-TP refolded and purified from inclusion bodies.** Lane 1: Inclusion bodies; lane 2: PC-TP pool after Ni^{2+} high bond fractionation.

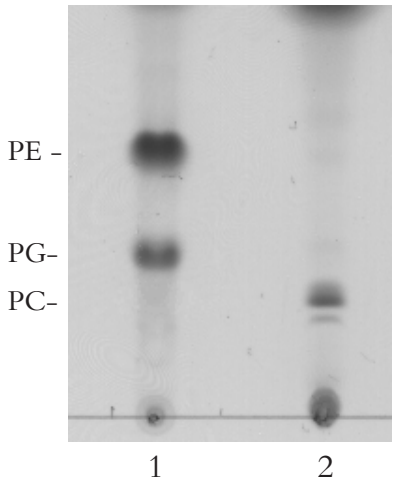

Figure 5. **Determination of the phospholipid content of recombinant PC-TP before and after incubation with PC vesicles by high performance thin layer chromatography.** Lane 1: lipid extract from recombinant PC-TP; lane 2: lipid extract from recombinant PC-TP after incubation with PC vesicles. Phospholipids were detected by staining with iodine vapour.

Composition of the phospholipids bound

PC-TP from bovine liver contains PC in a 1:1 stoichiometric complex (1). Since *E. coli* membranes lack PC, it was determined whether recombinant PC-TP has bound other phospholipids. After extraction it was established by HP-TLC that PC-TP contains PE and PG (figure 5; lane 1). Based on phosphorus determination, it was estimated that each PC-TP molecule contained one phospholipid molecule and that PE and PG were present in a molar ratio of 2.4 to 1. This ratio resembles the abundance of these phospholipids in E. coli membranes (25). After incubation of PC-TP with egg PC vesicles, the bound PE and PG were completely exchanged for PC (figure 5; lane 2).

In previous studies it was found that PC-TP has a preference for unsaturated PC species (20, 26-29). Analysis of the lipid extract by FAB-MS showed that PC-TP contained four PE species and three PG species (table 2). As one can see, the molecular species composition of both PE and PG bound to PC-TP differed significantly from that of the *E. coli*. PC-TP has a clear preference for the di-monounsaturated species as compared to the saturated species.

Table 2. **Molecular species composition of PE and PG in PC-TP and in *Escherichia coli* membranes**. N.d. is not detectable.

Phospholipid	*sn*-1, *sn*-2	Composition of species bound by PC-TP	Composition of species in *E. coli*[*]
		(mol% of total species)	
PE	16:0, 16:0	n.d.	7
	16:0, 16:1	32	31
	16:0, 17:cy	n.d.	19
	16:0, 18:1	28	23
	18:1, 16:1	23	5
	18:1, 18:1	17	2
	Other species	n.d.	13
PG	16:0, 16:0	n.d.	10
	16:0, 16:1	32	24
	16:0, 17:cy	n.d.	10
	16:0, 18:1	47	34
	18:1, 16:1	n.d.	3
	18:1, 18:1	21	6
	Other species	n.d.	13

[*]Data according to Wilkinson *et al.* (25).

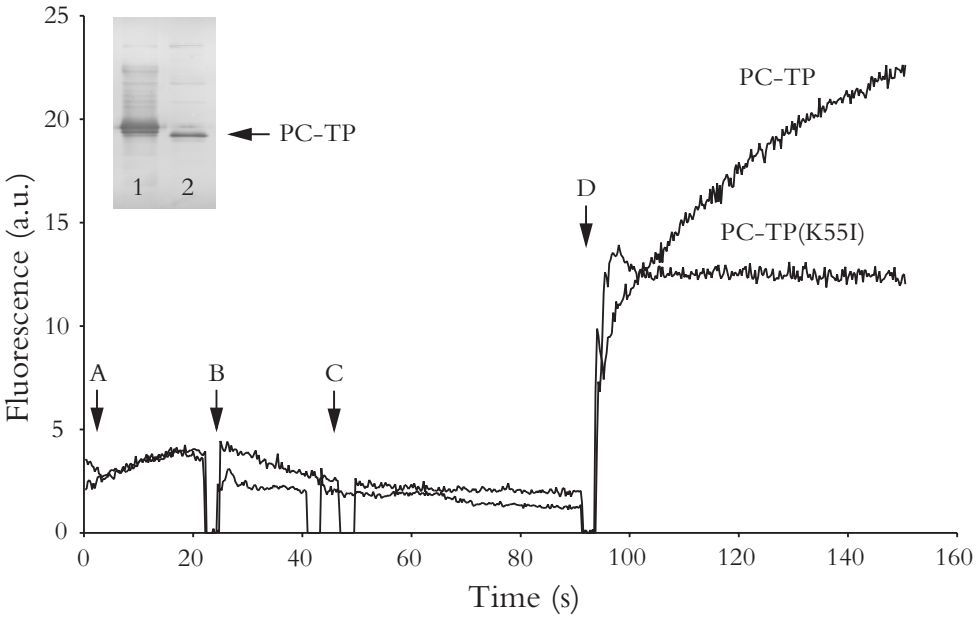

Figure 6. **Effect of the replacement of Lys^{55} with Ile on PC transfer activity.** *E. coli* cytosol (1.5 mg of protein) was tested for PC transfer activity in a continuous fluorescence transfer assay. Normal bovine PC-TP expressed in *E. coli* was used as control. Arrows indicate the addition of the donor vesicles (A), the acceptor vesicles (B), bovine serum albumin (C) and the cytosol (D). The inset shows the Western blot analysis of the cytosolic fractions. Lane 1: 20 μg PC-TP cytosol; lane 2: 20 μg PC-TP (K55I) cytosol.

The role of Lys^{55} in PC transfer

Modification of Lys^{55} by phenylisothiocyanate inhibited PC-TP from bovine liver (12). It was proposed that this lysine is part of the peptide segment that interacts with the membrane. Accordingly, based on the structural analogy with steroidogenic acute regulatory protein (StAR) Lys^{55} is located in a β-strand (β3) which forms part of the hydrophobic lipid binding site (5). However, phenylisothiocyanate is a bulky group as compared to the lysine residue, which could limit the physiological significance of this observation. The role of Lys^{55} was further studied by site-directed mutagenesis replacing Lys^{55} with Ile. As shown in figure 6, the *E. coli* cytosol containing the mutated PC-TP (K55I) did not express any PC transfer activity as compared to the control.

Discussion

In a previous study it was reported that bovine PC-TP could not be expressed in *E. coli* (14). It was inferred that the expression of PC-TP required PC, and that the absence of PC in these bacteria was the limiting factor. Our results refute this hypothesis as we observe an effective expression of bovine PC-TP in *E. coli*. From the cytosol fraction of a 10 l bacterial culture up to 100 mg of homogeneous His-tag PC-TP could be isolated. Recently, human PC-TP was effectively expressed in *E. coli* (10). This expression was 3-fold enhanced by redesigning the open reading frame according to preferred codon usage for *E. coli*. Under the latter conditions 10 mg cytosolic protein yielded 0.5 mg pure PC-TP as compared to 0.45 mg in our study (table 1). This indicates that the open reading frame of bovine PC-TP does not need to be remodelled to reach adequate expression levels in *E. coli*. The codon adaptation index (CAI) predicts the likelihood of heterologous gene expression in a micro-organism based on relative synonymous codon usage (30). Genes with CAI values of ≤ 0.30 are poorly expressed, whereas highly expressed genes have CAI values of ≥ 0.46. Surprisingly, the CAI value of bovine PC-TP is 0.29. Although this is higher than that of human PC-TP (0.24), bovine PC-TP should still be expressed poorly. The CAI value of the other PC-TP clones originating from rat and mouse is 0.30. Expression of the murine PC-TP in *E. coli* was 8-fold lower than that of bovine PC-TP (data not shown). Taken together, redesigning the open reading frame of the various PC-TPs according to *E. coli* preferred codon usage is useful to express substantial amounts of protein in this prokaryote, but is not necessary in all cases.

Here we also report that fully active PC-TP can be isolated from inclusion bodies. After folding, pure PC-TP was obtained in a one-step procedure. An essential component of the folding buffer was egg PC. Without this ligand no PC-TP transfer activity was recovered, strongly suggesting that PC be required to obtain the correct conformation during folding. Our observation that one mole of PE or PG is present per mole of recombinant PC-TP purified from cytosol, strongly suggests that these lipids can substitute for PC when PC-TP is expressed and folded in *E. coli*. Both *in vitro* and *in situ* phospholipids may be required to shield hydrophobic segments of PC-TP from the aqueous environment so as to prevent aggregation and to allow the protein to adopt the proper tertiary structure. Recently, phospholipids were also shown to be essential in the folding of phosphatidylinositol transfer protein isoforms from inclusion bodies (23).

Although the crystal structure of PC-TP has not yet been elucidated, PC-TP is representative for the START domain found in a number of lipid transporting

proteins among which the steroidogenic acute regulatory protein is the most prominent one (5). Based on the crystal structure of the START domain of human MLN64, it was proposed that PC-TP has a hydrophobic tunnel in which PC is accommodated with the polar head group interacting with Arg^{78} and Asp^{82} present in a loop at the surface of the protein. Here we demonstrate that PC-TP looses its activity when Lys^{55} is replaced with Ile. From the crystal structure we can deduce that Lys^{55} is present in the β3-strand which forms part of the lipid-binding site. Upon interaction of PC-TP with the interface, the lipid-binding site is believed to be exposed to the membrane (5). At this point the positively charged lysine residue could be needed to neutralise the negatively charged polar head groups of the phospholipids, thus enabling the safe passage of the acyl chains of PC. Expression of mutant PC-TPs in *E. coli* offers the possibility to further clarify the mechanism by which PC-TP inserts and extracts PC from a membrane.

Acknowledgement

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Chapter 3

Determinants of the phosphatidylcholine species affinity of the phosphatidylcholine transfer protein

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Abstract

Phosphatidylcholine transfer protein (PC-TP) is a water-soluble protein that catalyzes in vitro specifically the intermembrane exchange and net transfer of phosphatidylcholine (PC). Remarkably, PC-TP expressed in *Escherichia coli* contains PE and PG (chapter 2). Here we have replaced the endogenous PE and PG by PC by incubation with microsomal membranes. Relative to the microsomal PC species composition, PC-TP bound preferentially C16:0/C20:4-PC and C16:0/C18:2-PC (2-fold enriched) whereas the major microsomal species C18:0/C18:1-PC and C18:0/C18:2-PC were distinctly less bound. In these experiments, the PC species were analysed separately from the protein by positive ion fast atom bombardment (FAB-MS). The intact PC/PC-TP complex can be studied in the gas phase by nano-electrospray ionization time-of-flight mass spectrometry (ESI-TOF). The stability of these complexes in the gas phase was determined by elevating the cone voltage (CV) resulting in the appearance of the protein void of lipid. PC-TP containing a PC species carrying a palmitoyl chain on the *sn*-1 position was less stable than PC-TP containing a PC species carrying a stearoyl chain given that these complexes were dissociated for 50% at a CV of roughly 30 V and 45 V, respectively. The acyl chain on the *sn*-2 position did not have an effect on the stability of the complex. Taken together, these results indicate that the uptake of PC species by PC-TP is regulated by the ease with which molecular species of PC leave the (microsomal) membrane. The release could be determined by the interaction between PC-TP and PC as was shown by the ESI-TOF measurements. Higher uptake and release rates of C16:0/PUFA-PC eventually lead to accelerated exchange of especially this species.

Introduction

PC-TP is a water-soluble protein that catalyzes *in vitro* specifically the intermembrane exchange and net transfer of PC (1, 2). PC carried by PC-TP resides in an enclosed internal cavity completely shielded from the medium (3-6). Time-resolved fluorescence measurements using fluorescently labelled PC, showed that the *sn*-1 and *sn*-2 PC acyl chains are positioned separately in the lipid binding site (7). These binding pockets are believed to have different properties, exemplified by their acyl chain preference site (7) (8, 9). By a competition-binding assay it was established that PC-TP has the highest affinity for a C16:0 on the *sn*-1 and a polyunsaturated fatty acids (PUFA) on the *sn*-2 position of PC (10). PC species extracted from PC-

TP isolated from bovine liver are enriched in the same species (chapter 1). Specifically C16:0/PUFA PC-species are known to be secreted by the liver into bile (11-14) or as component of high-density lipoprotein (15). Furthermore, these PC species are substrate for cytosolic phospholipase A_2 (PLA_2)(16-20) releasing PUFAs that are well-known precursors in the eicosanoid synthesis (21). In these processes, PC-TP may be involved in transporting PC from its site of synthesis to its site of processing.

Thus far, the PC species affinity of PC-TP has been assessed in model systems consisting of PC vesicles or monolayers. However, since the source of *de novo* synthesized PC is the endoplasmic reticulum (22), we have studied the binding of PC species to PC-TP using bovine liver microsomes as donor membranes. The microsomal fraction of liver tissue consists mainly of rough and smooth endoplasmic reticulum, supplemented by the Golgi membranes and the plasma membrane (23). In these experiments, the PC species were analysed separately from the protein by positive ion FAB-MS. To study the protein-lipid interaction in depth we have analysed intact PC/PC-TP in the gas phase by nano-ESI-TOF. ESI-TOF has been used to study non-covalent interactions of intact proteins with metals, ligands, peptides, oligonucleotides, DNA and other proteins (24-26). By adjusting the CV, the gas phase protein-lipid complexes were dissociated. The extent of dissociation differed clearly for the various PC species bound by PC-TP.

Experimental procedures

Materials

Native PC-TP was isolated from bovine liver as described by Westerman *et al.* (27). Recombinant PC-TP was isolated according to chapter 2. PD-10 columns were purchased from Amersham Pharmacia Biotech AB (Uppsala, Sweden). The allsphere silica column (100 x 6.4 mm; 3 μm particles) was purchased from Alltech (Breda, The Netherlands). 10 and 100 kDa cut-off filters were obtained from Amicon (Beverly, USA). Phosphatidic acid (PA), egg yolk PC, C16:0/C18:1-PC, C16:0/C20:4-PC, C18:0/C18:1-PC, C18:0/C20:4-PC were purchased from Sigma (ST. Louis, USA).

Preparation of microsomes and PC/PA vesicles

Microsomes were prepared from bovine liver according to De Duve (28). The microsomal pellet was resuspended in 0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl pH 7.4 (SET buffer) and the suspension stored at –20°C until use. Peripheral

proteins were removed by washing the suspension two times with 10 mM Tris-HCl pH 8.6. After centrifugation the pellet was resuspended in SET buffer. Microsomal phospholipids were extracted from the microsomes by the method of Bligh and Dyer (29). Subsequently, microsomal PC was purified by high performance liquid chromatography (HPLC) using an allsphere silica column (100 x 6.4 mm; 3 μm particles) according to Ellingson *et al.* (30). Small unilamellar vesicles (SUVs) consisting of microsomal phospholipids or PC and eggPA (90/10 mol%) were prepared by ethanol injection (20 mM solution) (31) in 10 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM EDTA.

Incubation of PC-TP with microsomes and vesicles

PC-TP (30 nmol) was incubated with PC/PA vesicles (3 μmol PC) or with microsomes (3 μmol PC) in 1 ml 10 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM EDTA by slowly rotating the mixture for 1 h at room temperature (RT). At the end of incubation PC-TP was separated from the PC/PA vesicles using a 100 kDa cut-off filter. Control experiments without PC-TP showed that the vesicles were completely retained by the filter since no phospholipid was detected in the filtrate. Microsomes were removed from the incubation mixture by pH 5.1 precipitation and centrifugation at 13,000 x *g* for 10 min at 4°C. To ensure complete removal of microsomes, the ensuing supernatant was centrifuged at 250,000 x *g* for 1 hr at 4°C. Control experiments without PC-TP indicated that no PC was present in the cleared solution.

Preparation of phospholipid-transfer protein complexes

PC (1 μmol) was dried under nitrogen and dissolved in 40 μl ethanol. SUVs in 10 mM Tris-HCl pH 7.4, 10 mM β-mercaptoethanol were prepared as described above. PC-TP (10 nmol) in 10 mM Tris-HCl pH 7.4, 10 mM β-mercaptoethanol (1.5 ml) was incubated with the PC vesicles for 1.5 hrs at RT. The protein was separated from the vesicles as described above.

Electrospray time-of-flight analysis of protein-lipid complexes

Prior to analysis, PC-TP was concentrated to 2 mg/ml on 10 kDa cut-off filters and then diluted 10 times with 50 mM ammoniumacetate, pH 7.4 to a final concentration of approximately 7 mM. Mass spectra were recorded using a quadrupole time-of-flight instrument (Micromass Ltd., Manchester, UK) operating in the positive ion mode equipped with a nano-electrospray source. The potential between the nano-electrospray needle and the sample cone was set at 1500 V and the

cone voltage was varied between 20 and 120 V. Nano-electrospray needles were made from borosilicate glass capillaries with a P-97 puller (Sutter Instrument Co., Novato, USA). Needles were gold coated using an Edwards Scancoat 818 sputter coater (Crawley, UK). Dissociation of the complex was calculated by the following formula: 100% x area of combined complex peaks/(area of combined complex peaks + combined protein void of PC peaks).

Lipid analysis

Lipids were extracted from PC-TP by the method of Bligh and Dyer (29). PC molecular species were analysed by fast atom bombardment and tandem mass spectrometry as described by Geijtenbeek *et al.* (32) or by HPLC as described by Brouwers *et al.* (38).

Results

FAB-MS vs. HPLC

Although FAB-MS has been used for quantitative analysis of PC molecular species (32), there is considerable evidence that the acyl chain lengths influences the PC responsive peaks (33, 34). The difference between the natural occurring PC species in liver seems to be marginal, but could disturb our results. Therefore, we analyzed the molecular species composition of microsomal PC by positive ion FAB-MS and HPLC (table 1). Both techniques gave comparable results showing that the major species are C18:0/C18:1-PC (25%) and C18:0/C18:2-PC (25-30%). The difference between the C18:0/C20:4-PC abundance as determined with FAB-MS

Table 1. **The molecular PC species composition of bovine liver microsomes.**

sn-1, *sn*-2	Composition as determined by FAB-MS	Composition as determined by HPLC
	(mol% of total species)	
16:0, 18:1	13.0 ± 0.7	12.9 ± 0.4
16:0, 18:2	8.9 ± 0.3	10.7 ± 0.4
16:0, 20:4	7.7 ± 1.2	6.5 ± 0.4
18:0, 18:1	25.0 ± 1.6	25.9 ± 0.4
18:0, 18:2	25.9 ± 1.5	31.5 ± 1.2
18:0, 20:4	19.5 ± 1.5	12.6 ± 0.4

or HPLC is probably caused by natural variance, since with C16:0/C20:4-PC there is no significant difference in response.

Binding of PC species

Despite its specificity for PC, PC-TP discriminates between positional isomers (8, 10). Here we have measured the binding of PC species from microsomal membranes to PC-TP. Analysis by FAB-MS showed that relative to the PC composition of the microsomes, the PC bound to PC-TP was 2-fold enriched in C16:0/C20:4 and C16:0/C18:2-PC, whereas the major microsomal species C18:0/C18:1 and C18:0/18:2-PC were distinctly less bound (figure 1). A similar binding profile was obtained when PC-TP was incubated with vesicles containing microsomal PC. Incubation of PC-TP with microsomes lacking peripheral proteins or with vesicles

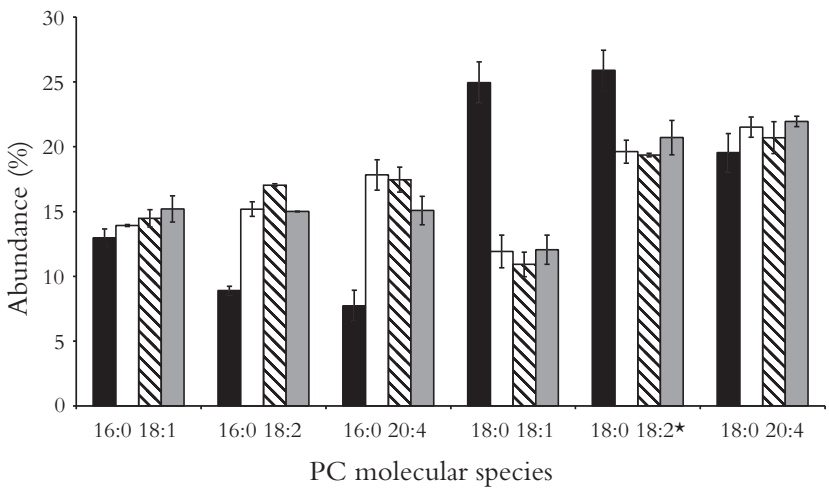

Figure 1. **Binding of PC molecular species to native PC-TP and recombinant PC-TP.** Shown is the abundance of PC species in 1) bovine liver microsomes (black bars), 2) bound by native (white bars) or recombinant (hatched bars) PC-TP incubated with bovine liver microsomes, and 3) bound by native PC-TP incubated with PC/PA vesicles (grey bars). *C18:0/C18:2-PC has the same molecular weight as C18:1/C18:1-PC and can not therefore be distinguished using FAB-MS. By HPLC it was estimated that the abundance of C18:1/C18:1-PC bound to PC-TP is less than 10 % of the total of the two species.

containing microsomal phospholipids gave rise to comparable PC species profiles (results not shown). This indicates that microsomal membrane proteins and other lipid classes had no effect on the binding preference of PC-TP. The same experiments were performed using recombinant PC-TP. Despite the six additional amino acid residues on the N-terminus (chapter 2), the PC species profiles were similar.

ESI-TOF analysis of PC-TP

Nano-electrospray ionization time-of-flight mass spectrometry was used to analyse the intact C18:0/C18:1-PC/PC-TP complex (figure 2). At a cone voltage of 50 V, free PC (m/z 788), three peaks representing the $[M+10H^+]^{10+}$ (m/z 2474), $[M+9H^+]^{9+}$ (m/z 2749) and $[M+8H^+]^{8+}$ (m/z 3093) of the protein void of PC, and two peaks representing the $[M+10H^+]^{10+}$ (m/z 2553) and $[M+9H^+]^{9+}$ (m/z 2835) of the PC/PC-TP complex were detected. The difference in average mass calculated from the three charged states of the PC/PC-TP complex (25520 ± 4 Da) and the free protein (24737 ± 5 Da) correlates with the mass of C18:0/C18:1-PC (788 Da). Close examination of the ion signals revealed several additional peaks. Hence, to facilitate the interpretation of the data, the charge state spectrum was deconvoluted

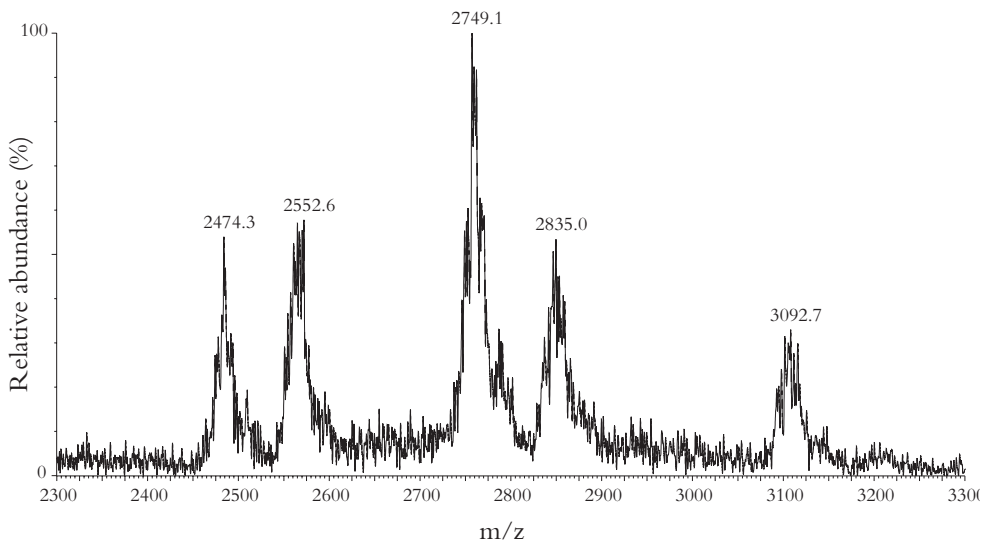

Figure 2. **Nano-ESI-TOF mass spectrum of the C18:0/C18:1-PC/PC-TP complex.** The cone voltage is set at 50 V dissociating approximately half the complex. PC-TP void of PC is visible at m/z 2474 ($[M+10H+]^{10+}$), 2749 ($[M+9H+]^{9+}$) and 3092 ($[M+8H+]^{8+}$). The complex can be found at m/z 2552 ($[M+10H+]^{10+}$) and 2835 ($[M+9H+]^{9+}$).

to a neutral mass spectrum using MaxEnt (figure 3). The peaks representing the complex and the free protein were clearly separated. Each peak was composed of three major peaks, each of which again composed of three minor peaks. The difference between each of the major peaks is about 76 Da indicating that the β-mercaptoethanol has reacted with a cysteine of PC-TP. The presence of this reaction product also explains the mass of the first major peak (i.e. 24737 Da), which differs approximately 76 Da from the calculated mass of PC-TP (i.e. 24655 Da). The difference in mass (22 Da) between the minor peaks can be accounted for by the presence of sodium. As shown in figure 3, the dissociation of the C18:0/C18:1-PC/PC-TP complex increases with the voltage applied to the cone. By increasing the voltage from 20 to 90 V, the dissociation of the complex increased from 15 to 100 % (panel a-d).

Interaction of PC molecular species with PC-TP as assessed by ESI-TOF

PC-TP prefers PC species carrying a C16:0 fatty acid on the *sn*-1 position as compared to a C18:0 fatty acid and PUFAs on the *sn*-2 position (see above)(10). Using ESI-TOF analysis of intact protein-lipid complexes we have examined PC-TP containing C16:0/C18:1-PC, C16:0/C20:4-PC, C18:0/C18:1-PC and C18:0/C20:4-PC. The areas of the combined peaks were used to follow the dissociation as a function of the cone voltage. As shown in figure 4, the PC binding

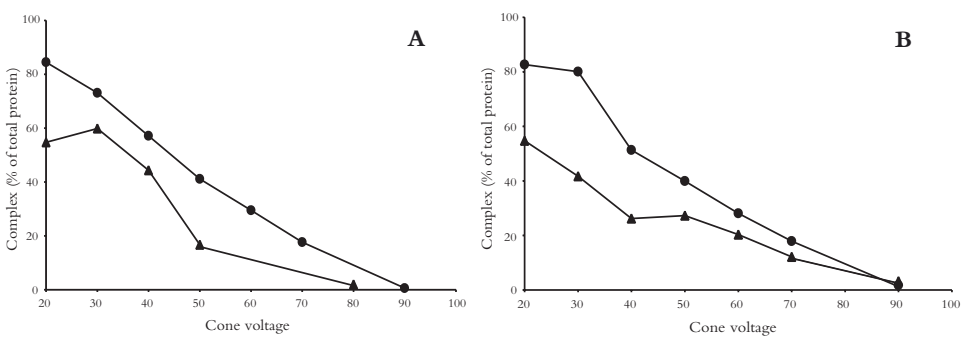

Figure 4. **Dissociation of PC/PC-TP complexes caused by elevation of the cone voltage.** Panel A: the dissociation of C18:0/C18:1-PC/PC-TP (circles) and C16:0/C18:1-PC/PC-TP (triangles). Panel B: the dissociation of C18:0/C20:4-PC/PC-TP (circles) and C16:0/20:4-PC/PC-TP (triangles).

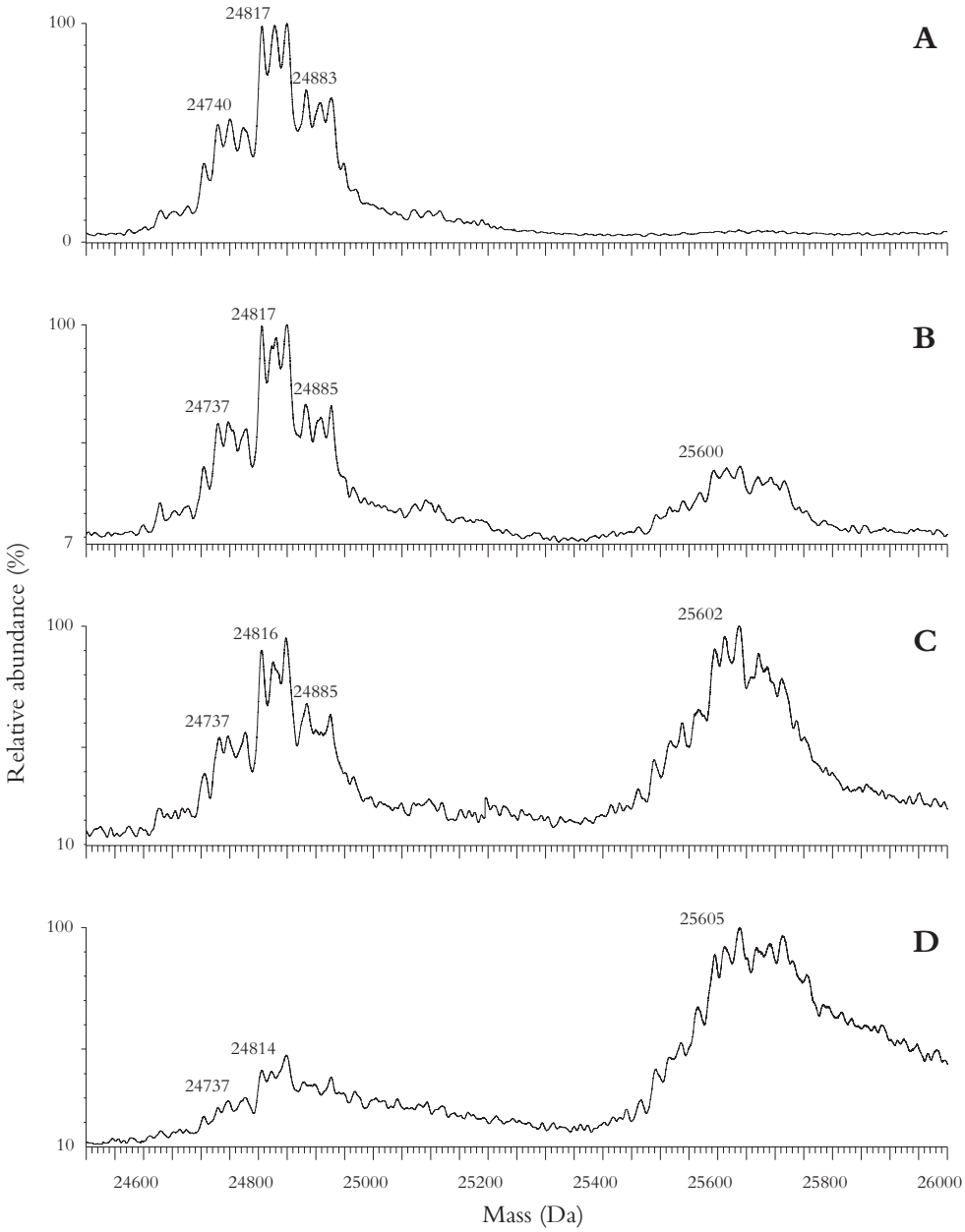

Figure 3. **Transformed data from nano-ESI-TOF mass spectra of the C18:0/C18:1-PC/PC-TP complex.** Panel A: dissociation of the PC/PC-TP complex at a cone voltage of 90 (A), 50 (B), 40 (C) and 20 V (D). The peak at 24817 Da represents the protein void of PC and the peak at 25602 Da the complex.

PC species carrying a C16:0 fatty acid was less stable than PC containing species carrying a C18:0 fatty acid. At a CV of 35, 50 % of the C16:0/C18:1-PC/PC-TP complex was dissociated, while a CV of 45 was required to release half of the C18:0/C18:1-PC (figure 4a). In the case of C16:0/C20:4-PC the required cone voltage was 25 as compared to 40 for the C18:0/C20:4-PC (figure 4b). Taking all cone voltages into consideration, the acyl chain on the *sn*-2 position (C18:1 or C20:4) does not seem to have an effect on the stability of the complex. The effect of CV on the extent of dissociation was reproducible.

Discussion

To investigate the interaction of PC-TP with PC in the presence of cellular membranes and all its components, we incubated PC-TP with microsomes, microsomes lacking the peripheral proteins, microsomal phospholipid vesicles and microsomal PC vesicles. In all cases, we observed that PC-TP preferentially binds PC with a C16:0 on the *sn*-1 position and a C18:2 or C20:4 on the *sn*-2 position. This is in good agreement with previous studies based on competition binding assays using fluorescent PC (10). It has been argued that the PC species preference reflected the capability of PC-TP to accommodate the *sn*-1 and *sn*-2 acyl chains in the corresponding pockets of the lipid-binding site (7-9). On the other hand, the binding preference may also reflect the ease with which molecular species of PC leave the membrane when PC-TP interacts with the interface. In contrast to PC in vesicles, PC in microsomes is known to rapidly equilibrate between the outer and inner leaflet most likely due to an intrinsic flippase (35). This and other microsomal proteins could possibly affect the binding of PC to PC-TP. However under the equilibrium binding conditions used in the present study, there was no marked discrepancy between the PC binding profile of PC-TP incubated with microsomes or with microsomal PC vesicles. Thus, proteins in the microsomal membrane have no effect on the PC species preference of PC-TP.

PC-TP complexed to phospholipid monomers can be analysed by nano-electrospray ionization time-of-flight mass spectrometry. The intact protein/lipid complex could easily be distinguished from the protein void of phospholipids. Being a zwitterionic phospholipid, PC can be protonated and thus also identified by ESI-TOF. As far as we know, this is the first time that such complexes were analysed by mass spectrometry. This opens new perspectives for establishing whether lipids bound to these transfer proteins are substrates for metabolism. In addition, it is conceivable

that ESI-TOF is amendable to study the organisation and metabolism of more complicated lipid/protein complexes, like serum lipoproteins.

The gas phase stability of the PC/PC-TP complex was studied by adjusting the voltage applied to the cone. The interaction of PC with PC-TP differed between PC species. C16:0/C18:1-PC and C16:0/C20:4-PC was released at a lower cone voltage then PC species containing a C18:0 acyl chain on the *sn*-1 position. On the other hand, the release was barely affected by the fatty acid on the *sn*-2 position. Given that specific binding sites exist for the *sn*-1 and *sn*-2 acyl chain (7-9), it appears that being accommodated in the hydrophobic binding site, the *sn*-1 saturated acyl chain dictates the stability of the complex more prominently than the *sn*-2 unsaturated acyl chain. In interpreting the results from ESI-TOF analysis of intact lipid-protein complexes, it should be noted that the dissociation of the complexes is carried out in the gas phase, whereas normally these complexes reside in an aqueous environment. However, we believe that the information obtained is relevant, since phospholipids bound to the transfer proteins are present in a highly hydrophobic environment completely shielded from water (4, 36, 37). This is supported by ESI-TOF analysis of PC/PI-TPα and PI/PI-TPα complexes, of which the latter is much more stable (data not shown) confirming the specificity of PI-TPα for PI as determined by biochemical methods (39-44).

As shown by incubation with microsomes, the preferred binding of C16:0/PUFA-PC at the membrane could reflect the capability of PC-TP to accommodate the *sn*-1 and *sn*-2 acyl chains in the corresponding pockets of the lipid-binding site. However, ESI-TOF analysis indicated that these same species are less strongly bound by PC-TP as compared to their C18:0 counterparts. Even more so, the acyl chain on the *sn*-2 position does not even affect binding strength. Thus, it seems more likely that the PC species preference of PC-TP is governed by the ease by which molecular species of PC leave the membrane when PC-TP interacts with the interface. Higher uptake and release rates eventually lead to accelerated exchange of especially C16:0/PUFA-PC species as mediated by PC-TP.

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Chapter 4

Clofibrate-induced relocation of the phosphatidylcholine transfer protein to mitochondria

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Abstract

The phosphatidylcholine transfer protein (PC-TP) is a specific transporter of phosphatidylcholine (PC) between membranes. In order to get more insight into its physiological function, we have studied the localization of PC-TP by microinjection of fluorescently labelled PC-TP in foetal bovine heart endothelial (FBHE) cells and by expression of an enhanced yellow fluorescent protein-PC-TP fusion protein in FBHE cells, human umbilical vein endothelial cells (HUVEC), HepG2 cells and NIH 3T3 mouse fibroblasts. Analysis by confocal laser scanning microscopy showed that PC-TP was evenly distributed throughout the cytosol with an apparently elevated level in nuclei. By measuring the fluorescence recovery after bleaching it was established that PC-TP is highly mobile throughout the cell, with its transport into the nucleus being hindered by the nuclear envelope.

Given the proposed function of PC-TP in lipid metabolism, we have tested a number of compounds (phorbol ester, bombesin, A23187, thrombin, dibutyryl cyclic AMP, oleate, clofibrate, platelet-derived growth factor, epidermal growth factor and hydrogen peroxide) for their ability to affect intracellular PC-TP distribution. Clofibrate (100 μM) was found to have an effect with PC-TP moving to mitochondria within 5 min of stimulation. Two other peroxisome proliferators, arachidonic acid (500 μM) and phytanic acid (10 μM), could cause this relocation as well. The relocation was restricted to the primary endothelial cells and did not occur with PC-TP(S110A) lacking the putative PKC-dependent phosphorylation site. In HepG2 cells and NIH 3T3 mouse fibroblasts the localization of PC-TP was not affected, possibly due to the fact that clofibrate does not induce PKC activation in these cells.

Introduction

PC-TP mediates *in vitro* specifically the intermembrane exchange and net transfer of PC (1, 2). Minor modifications of the head group of PC reduce or even abolish transport (1). Apart from its PC specificity, PC-TP distinguishes between PC positional isomers as well. By a competition-binding assay it was established that PC-TP has the highest affinity for a C16:0 on the *sn*-1 and polyunsaturated fatty acids (PUFAs) on the *sn*-2 position of PC (3). PC species extracted from PC-TP isolated from bovine liver are enriched in the same species (chapter 1). ESI-TOF analysis of the intact PC/PC-TP complex showed that the same species are bound less strongly

than PC species carrying a C18:0 on the *sn*-1 position (chapter 3). This led us to propose that that C16:0/PUFA-PC is taken up more readily due to the ease with which these molecular species of PC leave the membrane. These same species are then released more easily, resulting in enhanced PC-TP-mediated C16:0/PUFA-PC transfer.

The tissue distribution of PC-TP has been investigated thoroughly. In general, PC-TP is expressed in tissues exhibiting a high lipid turnover such as liver, kidney and intestine (4-6). Detailed studies assessing the subcellular localization using immunocytochemistry or immunofluorescence microscopy have not been performed as yet. Prediction of the subcellular localization with the *k* nearest neighbors classifier indicates that PC-TP resides in the cytoplasm (7, 8). Accordingly, about 60% of rat PC-TP is present in the cytosolic fraction after subcellular fractionation, the remainder being evenly distributed over the particulate fractions (4). A single washing step removed this protein, suggesting a dynamic equilibrium between membrane-bound and soluble PC-TP.

On bases of PC species preference and tissue distribution, a number of functions for PC-TP can be postulated, such as a role in bile and high-density-lipoprotein (HDL) formation or in hydrolysis of PC by phospholipases (see also chapter 1). In these cases, PC-TP is supposed to be involved in specific reactions instead of being a mere transporter of PC from the site of synthesis to other cellular membranes. Such a function should be reflected in a specific subcellular localization.

We have investigated the subcellular localization of PC-TP in FBHE cells, HUVEC, HepG2 cells and NIH3T3 mouse fibroblasts by expression of enhanced yellow fluorescent protein-PC-TP fusion proteins (EYFP-PC-TP). The distribution of PC-TP in FBHE cells was also studied by microinjection of Oregon Green labelled bovine PC-TP. Since PC-TP transfers specifically PC, compounds known to affect PC metabolism were tested for their ability to influence the subcellular distribution of PC-TP. We show that clofibrate redirects PC-TP to mitochondria in endothelial cells (FBHE cells and HUVEC), but not in HepG2 cells or mouse NIH3T3 mouse fibroblasts. This relocation is possibly related to phosphorylation of PC-TP.

Experimental procedures

Materials

FBHE cells were a gift from Mr. J.M. van Aken (Utrecht University, The Netherlands). HUVEC were isolated by Dr. G. Heijnen-Snyder (University Medical Centre Utrecht, The Netherlands). HepG2 cells were a gift from Dr. W. Stoorvogel (University Medical Centre Utrecht, The Netherlands). NIH3T3 mouse fibroblasts were a gift from Dr. P. Meijer, (Hubrecht Laboratorium, The Netherlands). The pEYFP-C1 vector was purchased from Clontech (Palo Alto, USA). The pBluescript SK^- vector and the Quickchange site-directed mutagenesis kit were obtained from Stratagene (La Jolla, USA). The pGEM T-easy vector was purchased from Promega (Madison, USA). Primers were synthesized by Eurogentec (Seraing, Belgium). The FuGENE6 Transfection Reagent Kit was obtained from Roche Molecular Biochemicals (Basel, Switzerland). PD-10 columns were purchased from Amersham Pharmacia Biotech AB (Uppsala, Sweden). 30 and 100 kDa molecular weight cut-off filters were from Amicon (Beverly, USA). Electroporation cuvette plus™ were obtained from Genetronics Inc. (San Diego, USA). G418 (geneticin), egg yolk PC, trinitrophenylphosphatidylethanolamine and phosphatidic acid (PA) prepared from egg yolk PC, 5-bromo-4-chloro-3-indoyl-phosphate *p*-toluidine salt (BCIP), *p*-nitro blue tetrazolium chloride (NBT), Coomassie Brilliant Blue R-250 and goat-anti-rabbit IgG conjugated with alkaline phosphatase (GAR-AP) were purchased from Sigma (ST. Louis, USA). LysoTracker Yellow and MitoTracker Red were obtained from Molecular Probes Europe BV (Leiden, The Netherlands). Antibodies against cytochrome C oxidase subunit IV were purchased from Research Diagnostics Inc. (Flanders, USA). Antibodies against nsL-TP labelled with cy3 were a kind gift from Dr. T.B. Dansen (University Utrecht, The Netherlands). 1-Palmitoyl-, 2-pyrenyl-decanoyl-PC was synthesised according established procedures and was a kind gift of Dr. P.J. Somerharju (University of Helsinki, Finland). Non-fat dry milk was purchased from Nutricia (Zoetermeer, The Netherlands).

Cell culturing

FBHE cells and HepG2 cells were grown in Dulbecco's modified Eagles medium (DMEM) containing 10% (v/v) foetal calf serum (FCS) buffered with 44 mM $NaHCO_3$. HUVEC were grown in RPMI-1640 medium containing 20% (v/v) human serum buffered with 44 mM $NaHCO_3$. NIH3T3 mouse fibroblasts were cultured in DMEM containing 10% (v/v) newborn calf serum (NCS). All cells were maintained at 37°C in a 7.5% CO_2 atmosphere and 95% humidity.

Gel electrophoresis and Western blotting

Cells grown in culture flasks were washed three times with phosphate-buffered saline (PBS; 8.3 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 6.9). Total cell lysates were obtained by lysis of the cells in 50 mM HEPES pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5 % NP-40, 0.5 % sodium deoxycholate, 0.1 % sodium dodecyl sulphate (SDS), 5 μg/ml leupeptin, 10 mM NaF. Subsequently, proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 12% acrylamide and 0.37% bis-acrylamide (9) and transferred to a nitrocellulose membrane (Schleicher & Schuell BA 85) by semi-dry Western blotting in a Multiphor II Nova Blot electrophoretic transfer unit (Pharmacia, Uppsala, Sweden) at 1 mA/cm^2 for 1 h at room temperature. The non-specific binding sites of the nitrocellulose membrane were blocked by incubating the membrane for 1 h in 2% non-fat dry milk (w/v) in 20 mM Tris-HCl pH 7.5, 0.5 M NaCl (TBS) at 37 °C. Subsequently, the blot was incubated for 1 h at 37°C with the affinity-purified antibody Ab270 or Ab6221 raised against native bovine PC-TP or truncated recombinant mouse PC-TP (affinity purified by Protein A Sepharose CL-4B) diluted 1:20 in TBS containing 0.2% non-fat dry milk (w/v). The blot was washed with TBS-Tween 20 (0.05% v/v) (3 x 10 min) and incubated for 1 h with GAR-AP diluted 1:5000 in TBS. The blot was washed again with TBS-Tween 20 (3 x 10 min). The immunoreactive proteins were visualized by incubating the blot in 10 ml 0.1 M $NaHCO_3$ pH 9.8, 10 mM $MgCl_2 \cdot 6H_2O$ containing 0.3 mg/ml BCIP and 0.15 mg/ml NBT as the colour development substrate.

Oregon Green labelling of PC-TP

Native and recombinant bovine PC-TP (without His-tag) (100 μg) were labelled with a 10-fold molar excess of freshly prepared Oregon Green Iodoacetamide 488 in PBS pH 8.5 in the presence of a 10-fold molar excess of the reducing agent tris-(2-carboxyethyl)phosphine. This mixture was incubated for 48 h under nitrogen at room temperature in the dark. The reaction was stopped by incubation with cysteine (1 μmol) for 30 min at room temperature. Excess label was removed using a PD-10 column equilibrated in PBS pH 7.4. Labelled protein was concentrated to 400 μg/ml using a 30 kDa molecular weight cut-off filter. By comparing the molar absorbance of the bound fluorophore and of PC-TP, it was estimated that one fluorophore was attached to one molecule of PC-TP. PC-TP transfer activity was measured according to van Paridon *et al.* (12).

Microinjection of Oregon Green-labelled PC-TP

FBHE cells were grown on glass cover slips to a density of 1 x 10^4 cells/cm^2. Two days prior to microinjection, the medium was replaced with DMEM containing 10% (v/v) FCS buffered with 20 mM Hepes pH 7.4. Fluorescently labelled PC-TP (400 μg/ml) was microinjected into the cytosol of FBHE cells by using a combination of an Eppendorf Microinjector (Model 5244) at 90 hPa pressure (approximately 0.2 s) and an Eppendorf Micromanipulator (Model 5170) under an inverted microscope with a 40x air objective. Cells were allowed to recover at 37°C for 1 h. Subsequently, the cells were analysed by confocal laser scanning microscopy (CLSM).

pEYFP-PC-TP constructs

Bovine PC-TP cDNA (10) was amplified by PCR using the following primers: 5'-cgc aag ctt cga att cc**a tg**g atc ctg ggg ccg gcg cct tc-3' (sense) and 5'-ctc gaa tcc ggt ac**c ta**g gtt ttc ttg tag ttc tac ga-3' (antisense). The start and stop codons are printed in bold. These primers introduce additional *Hind*III (sense) and *Kpn*I (antisense) restriction sites (underlined). Using these two enzymes PC-TP was cloned in frame into the pEYFP-C1 vector. Human PC-TP cDNA (11) was cloned into the pEYFP-C1 vector by the same cloning strategy. The primers used were: 5'-gcc tcg agc gaa ttc c**at g**ga gct ggc cgc cgg aag-3' (sense) and 5'-gag gcc ttc ggt acc **tta** ggt ttt ctt gag gta gt-3' (antisense). The start and stop codons are printed in bold. These primers introduce additional *Xho*I (sense) and *Kpn*I (antisense) restriction sites (underlined), with which PC-TP was cloned in frame into the pEYFP-C1 vector. Mouse PC-TP cDNA (11) was amplified by PCR using the following primers: 5'-gga tcc at**a tg**g cgg ggg ccg cat gct gct tc-3' (sense) and 5'-cgc ctc gag **tta** ggt ttt ctt gtg gta gtt ctg-3'. The start and stop codons are printed in bold. The PCR product was subcloned into the pGEM T-easy vector using the single 3'-t overhangs at the insertion site. By means of the *Apa*I and *Spe*I restriction sites in the pGEM T-easy multiple cloning site, the insert was transferred into the pBluescript SK^- vector. Subsequently, the mouse PC-TP cDNA insert was cloned in frame into the pEYFP-C1 vector using *Apa*I and *Xba*I. Proper insertion of all constructs was checked by restriction analysis and DNA sequencing. In addition, the complete EYFP-PC-TP (bovine, human and murine) open reading frame was cloned into the pET15b vector and expressed in *E. coli*. EYFP-PC-TP was isolated by IMAC (see chapter 2) and the transfer activity measured as described above.

Site-directed mutagenesis

Bovine PC-TP cDNA in the pEYFP-C1 vector was subcloned into the pBluescript SK$^-$ vector using *Bam*HI. Using the Quickchange site-directed mutagenesis method Ser^{110} was replaced with Ala. The following primers were used: 5'-cct ttt ccc atg **gct** aac aga gat tat gtt-3' (sense) and 5'-aac ata atc tct gtt **agc** cat ggg aaa agg-3' (antisense). The bold nucleotides encode the mutated amino acid (Ser^{110} to Ala) and simultaneously introduce a *Nco*I restriction site (underlined). Incorporation of the changed nucleotides into the construct was checked by restriction enzyme analysis and DNA sequencing. Subsequently, the mutated PC-TP(S110A) was cloned back into the pEYFP-C1 vector using *Bam*HI and the orientation checked by restriction analysis. Bovine PC-TP(S110A) was also expressed in *E. coli* using the pET15b expression vector. The PC transfer activity in the bacterial cytosol was measured as described above.

Transfection

FBHE cells, HepG2 cells and NIH3T3 mouse fibroblasts were transfected by lipofection. Cells were seeded at 1 x 10^4 cells/cm^2 on glass cover slips. After 24 h of growth, cells were transfected with 1 μg of the pEYFP-PC-TP vector using the FuGENE6 Transfection Reagent Kit according to the instruction of the manufacturer and after 24 h analysed by CLSM. HUVEC were transfected by electroporation. Cells were seeded at 2 x 10^4 cells/cm^2 in a 75 cm^2 flask. After 24 h of growth, the medium was removed and the cells washed two times with PBS. 0.25% Trypsin-0.03% EDTA (w/w)(1 ml) in PBS was added and the flask incubated at 37°C until the cells detached. Next, 5 ml RPMI medium containing 20% human serum was added and the cells centrifuged at 100 x *g* for 5 min. The cells were resuspended in 2 ml PBS and centrifuged again at 100 x *g* for 5 min. Subsequently, cells were resuspended in 0.8 ml PBS. DNA (4 μg) was added and the suspension was transferred into a 4 mm gap electroporation cuvette plus™. Cells were subjected to two pulses of 0.3 ms with a capacity of 25 μF and a voltage of 850 V using a Bio-Rad Gene pulser II (Bio-Rad Laboratories, Hercules, USA). Subsequently, 10 ml RPMI containing 20% human serum was added and the cells left to recuperate for 24 h under normal conditions on glass cover slips.

Stimulation of cells

Cells containing EYFP-PC-TP or Oregon Green labelled PC-TP, were grown to a density of 2 x 10^4 cells/cm^2 on glass coverslips and stimulated by fresh serum-free medium containing clofibrate and other agonists for different periods of time (0-120

min). The cells were fixed with 4% paraformaldehyde in PBS and the distribution of fluorescence was analysed by CLSM. In some instances, fluorescence was determined by *in vivo* imaging up to 30 min.

Identification of subcellular organelles

Cells were washed two times with PBS and fixed by incubation in 4% paraformaldehyde in PBS for 1 h at 37°C. Fixed cells were permeabilized by 0.5% Triton X-100 in PBS (5 min) and subsequently washed two times for 5 min in 50 mM NH_4Cl in PBS. Prior to labelling with antibodies, non-specific binding sites were blocked with 2 mg/ml gelatine in PBS for 1 h. Peroxisomes were identified by Cy3-labelled antibodies directed against the peroxisomal non-specific lipid transfer protein (nsL-TP) (13) and mitochondria by tetramethylrhodamine B isothiocyanate (TRITC)-labelled antibodies against cytochrome C oxidase subunit IV. The coverslips were mounted in 20 mM Tris-HCl pH 8.5, 24% Gelvatol 4-88, 6% glycerol. Mitochondria and lysosomes were identified in living cells by addition of serum-free medium containing 25 μM MitoTracker Red or LysoTracker Yellow.

Confocal laser scanning microscopy

Fluorescence was visualised by CLSM using a Leica TCSNT confocal laser scanning microscope on an inverted microscope DMIRBE (Leica Microsystems, GmbH, Heidelberg, Germany) with an argon-krypton laser as excitation source. In case of the Oregon Green 488 and EYFP, $\lambda_{excitation}$ and $\lambda_{emission}$ were 488 nm and 530 nm ± 15 nm, respectively. In case of Cy3, TRITC, MitoTracker Red and LysoTracker Yellow, $\lambda_{excitation}$ and $\lambda_{emission}$ were 568 nm and 600 nm ± 15 nm, respectively. Overlap from the green into the red channel and *vice versa* was checked before recording the images and, if necessary, corrected.

Bleaching

To bleach the fluorescently labelled PC-TP in cells, a distinct section of the cytosol or the nucleus was scanned 16 times with the pinhole set at maximum depth. Immediately after bleaching, settings were returned to normal and the remainder of the fluorescence visualized. To quantify the recovery after bleaching, the fluorescence intensity of each pixel in a part of the nucleus (2000 pixels) and the cytosol (14000 pixels) was determined at various time points and the fraction of fluorescence present in the nucleus calculated.

Correlation plots

Correlation plots were generated using the plotting macro provided with Scion Image release Beta 3b. A correlation coefficient of 1.0 implies an identical distribution, whereas a coefficient of 0.5 is indicative for a diffuse pattern showing no correlation at all.

Results

PC-TP levels and subcellular localization

The subcellular localization of PC-TP was investigated in four different cell lines, i.e. FBHE cells, HUVEC, HepG2 cells and NIH3T3 mouse fibroblasts. As established by densitometric scanning of immunoblots, it was estimated that FBHE cells, HUVEC and HepG2 cells contain 1.0, 0.1 and 3.4 μg PC-TP per mg total protein, respectively (figure 1). NIH3T3 cells do not contain detectable levels of PC-TP (results not shown). Affinity-purified polyclonal antibodies raised against bovine liver PC-TP were used to determine the intracellular distribution of PC-TP in FBHE cells, HUVEC and HepG2 cells. However, when prior to use the specific antibodies were removed by incubation with pure native bovine PC-TP, non-specific staining was still clearly visible (results not shown). These non-specific interactions prevented the use of the polyclonal antibody in the immunolocalization. Hence, the subcellular localization was determined by the microinjection of bovine PC-TP labelled with Oregon Green 488 and by the expression of an enhanced yellow fluorescent protein-PC-TP fusion protein (EYFP-PC-TP). Fluorescent tagging of PC-TP had no effect on the PC transfer activity (data not shown). The CLSM images in figure 2 show the localization of PC-TP in the equatorial plane. In the case of FBHE cells the Oregon Green labelled PC-TP and the EYFP-bovine PC-TP were found throughout the cytosol with elevated levels present in the nucleus (figure 2a-c). When EYFP-human

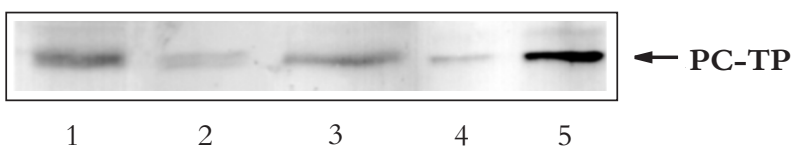
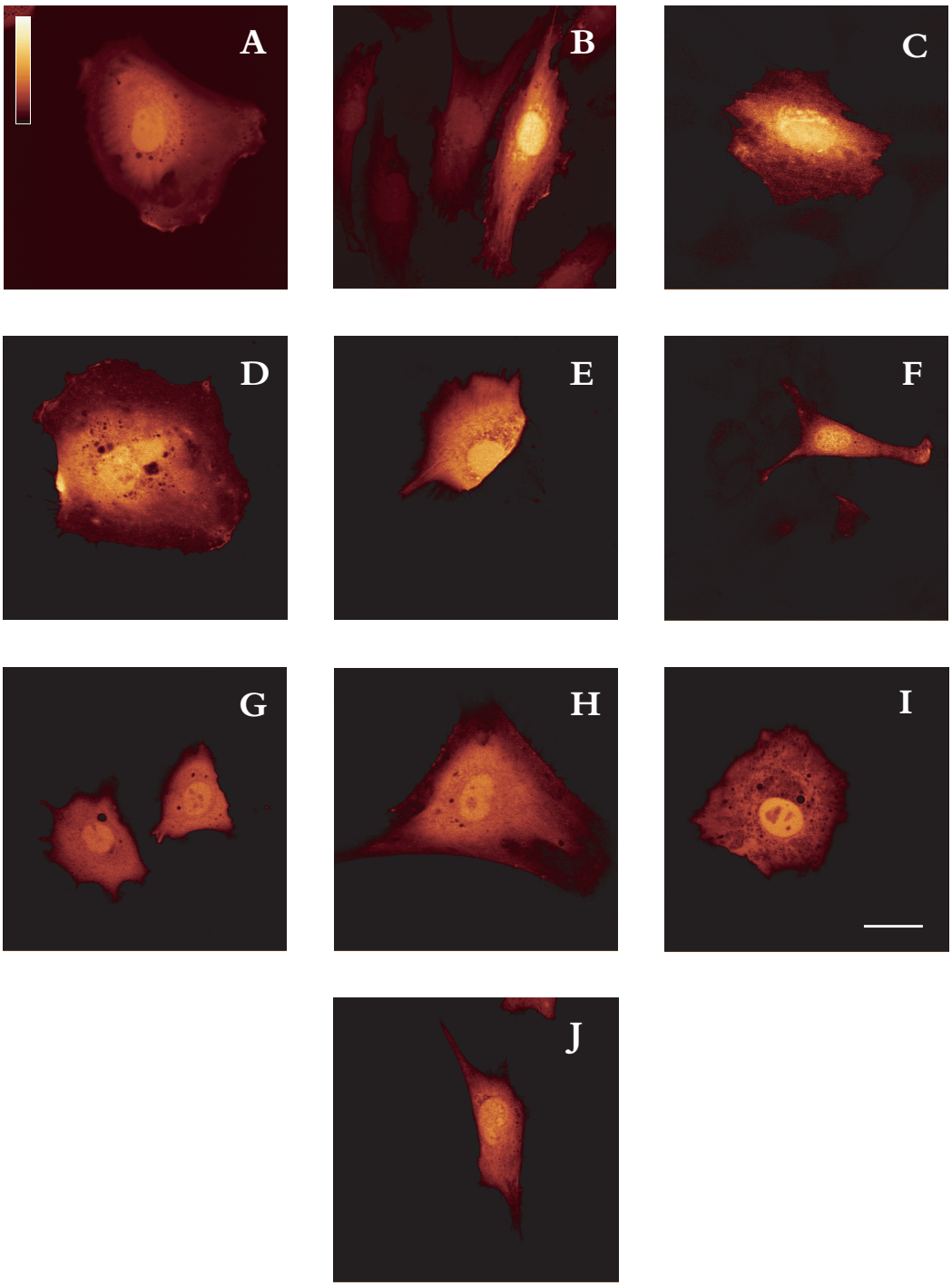


Figure 1. **Immunoblot analysis of PC-TP in total cell lysates.** Lane 1: HepG2 cells (20 μg protein). Lane 2: HUVEC (150 μg protein). Lane 3: FBHE cells (25 μg protein). Lane 4: native bovine PC-TP (10 ng). Lane 5: native bovine PC-TP (30 ng).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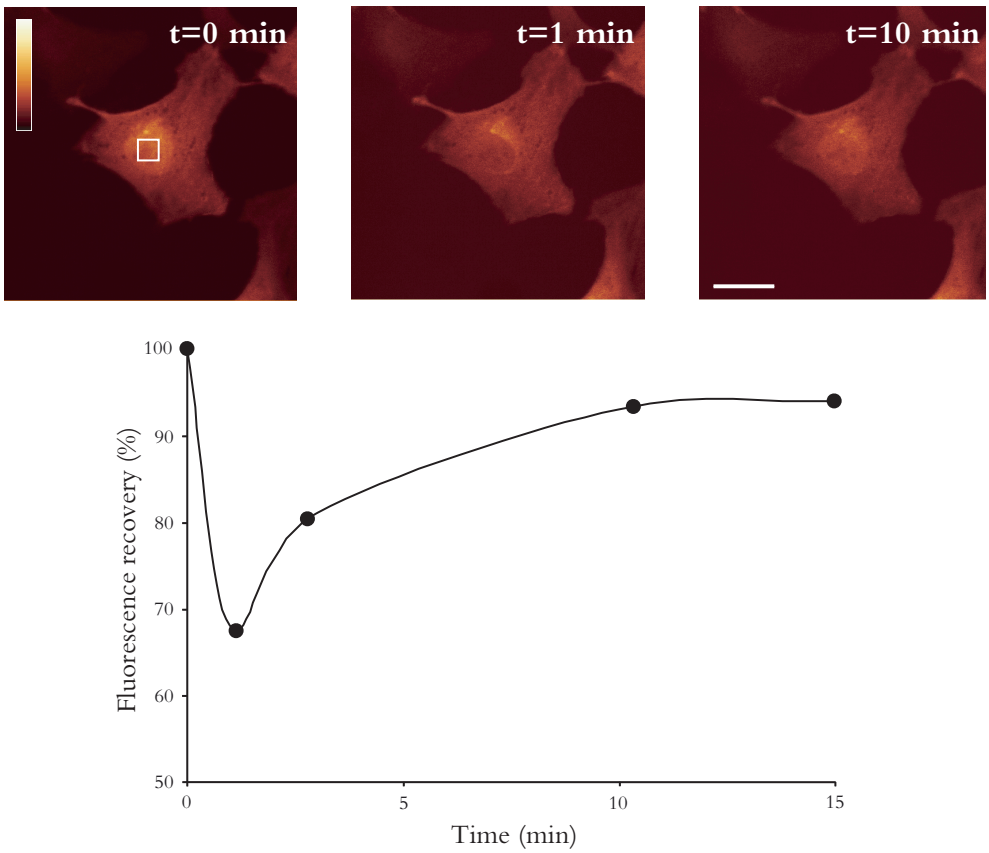


Figure 3. **Fluorescence recovery after bleaching of Oregon Green-labelled PC-TP in the nucleus of FBHE cells**. Upper panel: distribution of PC-TP at different time points. The white square indicates the bleached area in the nucleus. Inset shows the intensity from high (white) to low (black); the bar represents a length of 20 μm. Images were obtained by CLSM. Lower panel: quantification of fluorescence recovery.

◀Figure 2. **Subcellular distribution of PC-TP.** Panel A: Oregon Green-labelled PC-TP microinjected into FBHE cells; panel B: Oregon Green-labelled recombinant PC-TP microinjected into FBHE cells; panel C-F: EYFP-PC-TP expressed in FBHE cells, HUVEC, HepG2 cells and NIH3T3 mouse fibroblasts; panel G-J: EYFP expressed in FBHE cells, HUVEC, HepG2 cells and NIH 3T3 mouse fibroblasts. Inset shows the intensity from high (white) to low (black); the bar represents a length of 20 μm. Images were obtained by CLSM.

PC-TP was expressed in HUVEC and HepG2 cells or EYFP-murine PC-TP in NIH3T3 fibroblasts, the same localization was observed (figure 2d-f). Since pure human or murine PC-TP was not available, the localization studies of these cells were restricted to the fusion protein. Expression of EYFP in all four cell lines gave a similar distribution indicating that the enrichment of PC-TP in the nucleus was not an inherent property of this protein (figure g-j).

Intracellular mobility

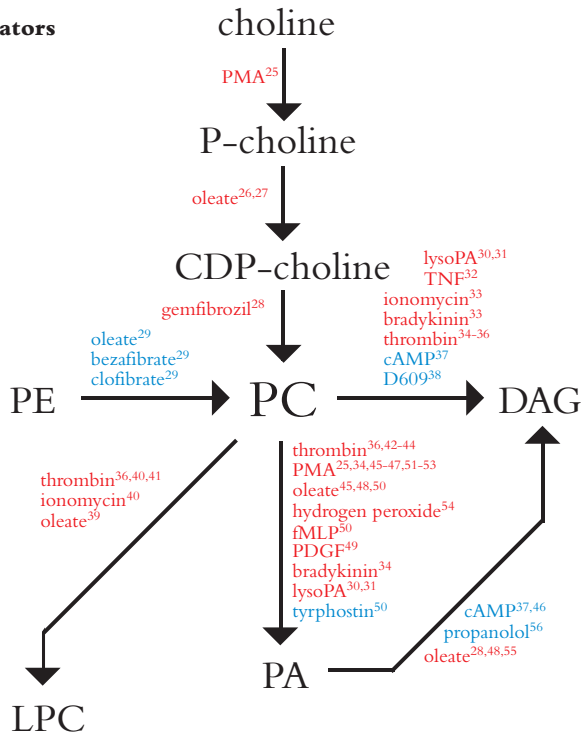
To investigate the mobility of Oregon Green-labelled PC-TP in the cell, the fluorophore was bleached in a distinct section of either the nucleus or the cytosol, and the recovery of fluorescence measured. Bleaching a section of the nucleus diminished the fluorescence intensity of the entire nucleus (figure 3). After bleaching the fluorescence intensity was restored to virtually the initial level within 10 min. The time course of recovery after bleaching in the nucleus is shown in figure 3 lower panel. In case of bleaching a section of the cytosol the fluorescence recovery was instantaneous (less than 30 sec). This indicates that PC-TP can move freely throughout the cell, yet that the movement across the nuclear membrane is restricted. Similar results were obtained by bleaching EYFP-PC-TP in FHBE cells, HUVEC and HepG2 cells. In case of the EYFP fusion proteins the fluorescence recovery in the nucleus was approximately 15 min (data not shown).

Clofibrate-induced relocation to mitochondria

With PC-TP being able to move freely through the cell, it can deliver PC to specific sites of metabolism or to distinct membrane domains in need of PC. To obtain evidence for this, FBHE cells microinjected with Oregon Green-labelled PC-TP were treated with compounds that affect PC hydrolysis or synthesis. The compounds tested were phorbol ester (50 and 100 ng/ml), bombesin (10 nM), A23187 (1 and 4 μg/ml), thrombin (0.1 and 1 U/ml), dibutyryl cyclic AMP (1 mM), oleate (0.5 and 2 mM), clofibrate (100, 200 and 500 μM), lysophosphatidic acid (10 μg/ml), tumor necrosis factor (1 and 50 ng/ml), platelet-derived growth factor (20 ng/ml), epidermal growth factor (50 ng/ml) and hydrogen peroxide (200 μM) (for a survey of compounds affecting PC metabolism see figure 4). None of these compounds influenced the distribution of fluorescently labelled PC-TP (data not shown). An exception however, was clofibrate, an inhibitor of the PE methylation pathway that caused an extensive redistribution of PC-TP within 5 min (figure 5). Up to 25 min PC-TP remained associated with distinct cellular structures. As a control Oregon Green-labelled bovine serum albumin was injected into FBHE cells. The distribution of this protein was not affected by clofibrate treatment (data not shown).

Since clofibrate primarily influences the function of peroxisomes (14, 15) and mitochondria (16-18), attention was focussed on these two organelles as possible sites of interaction for PC-TP. To be sure that labelling of these structures did not indicate enhanced degradation of PC-TP, lysosomes were also taken into consideration. After a 5 min-stimulation by clofibrate and fixation of the FBHE cells, PC-TP did not colocalize with anti-bodies directed against nsL-TP, a peroxisomal protein (figure 6a). Using a lysosomal marker (LysoTracker Yellow) during *in vivo* imaging, it was demonstrated that the PC-TP-associated structures were not lysosomes (figure 6b). However, a distinct colocalization was observed with MitoTracker Red (also measured *in vivo*) indicating that PC-TP is associated with mitochondria after clofibrate stimulation (figure 6c). 10 μM phytanic acid, which is another peroxisome proliferator (19, 20), induces PC-TP to move to mitochondria as well (figure 6d). This colocalization was confirmed by computing the correlation between the red and green channel. By superimposing the two images the intensity of each separate pixel was compared (figure 7). The correlation coefficient between MitoTracker Red and PC-TP after clofibrate- and phytanic acid-induced relocation was 0.92 and 0.90 respectively, whereas the correlation coefficients for peroxisomes and lysosomes were 0.56 and 0.54, respectively.

Figure 4. **Overview of activators (red) and inhibitors (blue) affecting PC metabolism.**



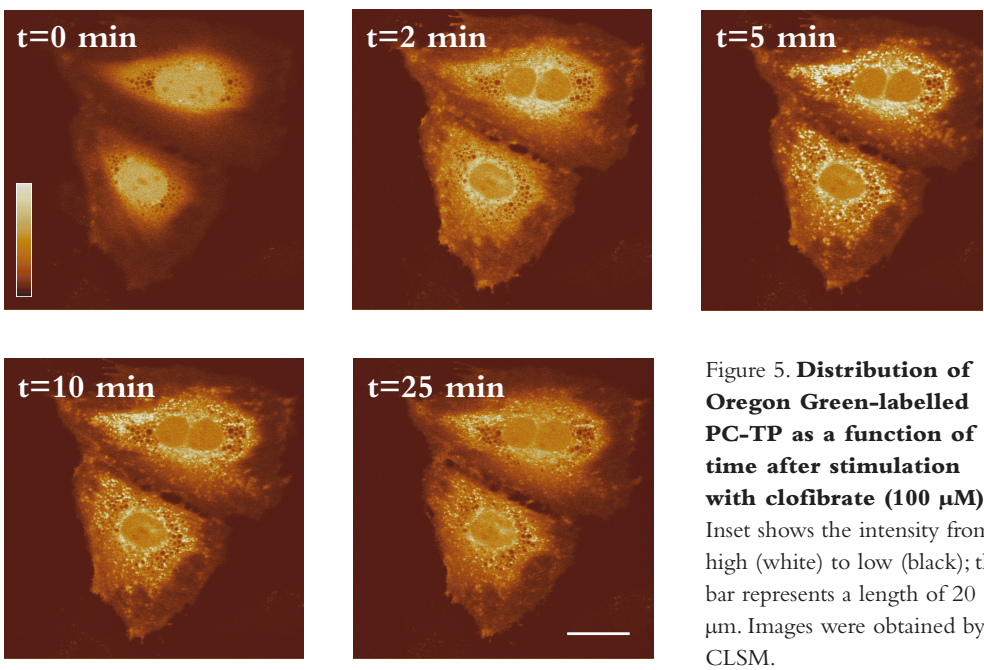


Figure 5. **Distribution of Oregon Green-labelled PC-TP as a function of time after stimulation with clofibrate (100 µM).** Inset shows the intensity from high (white) to low (black); the bar represents a length of 20 µm. Images were obtained by CLSM.

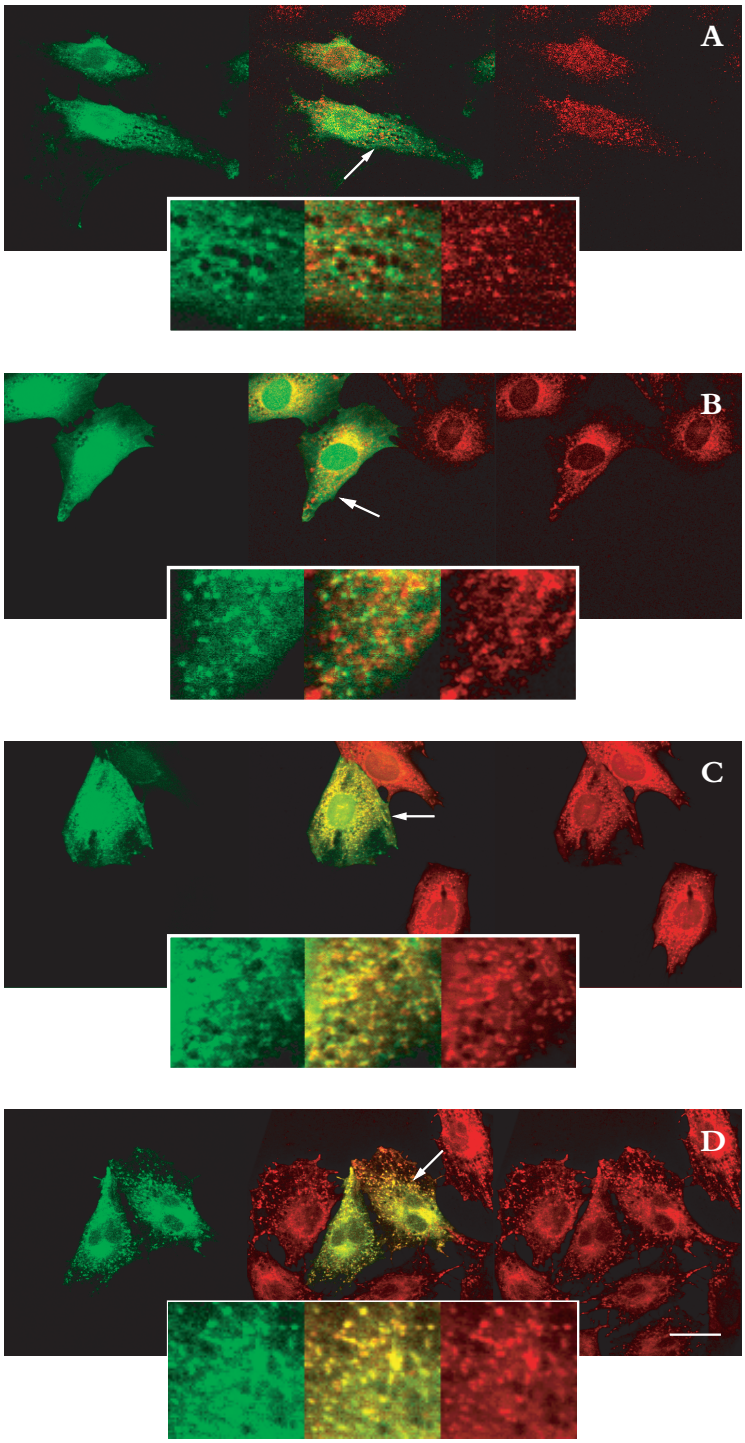


Figure 6. **Subcellular localization of Oregon Green-labelled PC-TP after stimulation with clofibrate or phytanic acid.** Panel A-C: after stimulation with 100 μM clofibrate; panel D: after stimulation with 10 μM phytanic acid. PC-TP is shown on the left and markers for peroxisomes (A), lysosomes (B) and mitochondria (C,D) on the right. The middle image of each panel shows the colocalization as indicated by the yellow colour. Peroxisomes were identified by Cy3-labelled antibodies directed against nsL-TP; lysosomes by LysoTracker Yellow; and mitochondria by MitoTracker Red. Arrows indicate the position of the inset; the bar represents 20 μm of length. All images were obtained by CLSM.

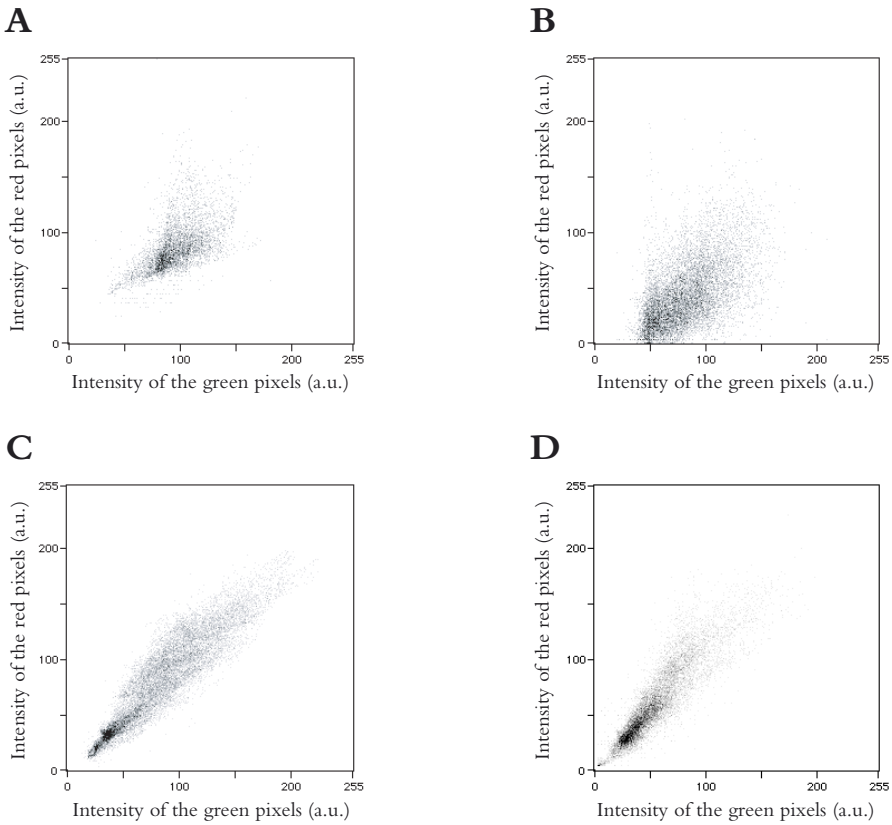
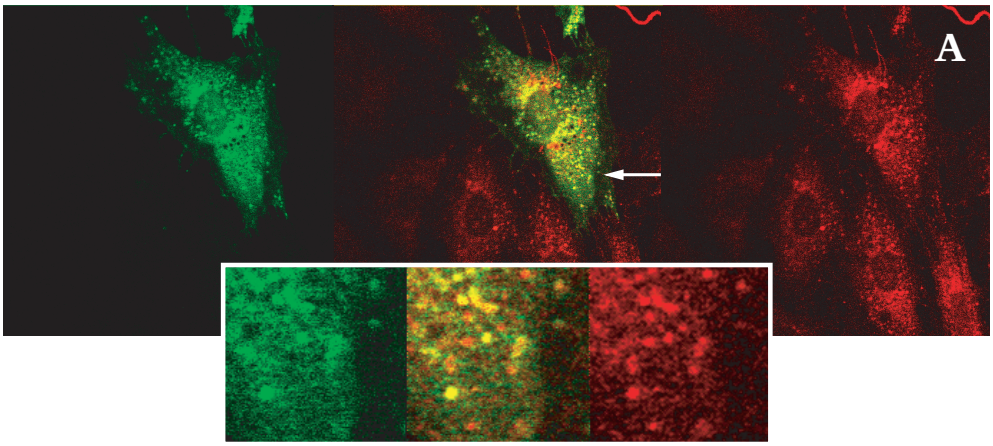


Figure 7. **Correlation plots of the subcellular distribution of PC-TP and markers of peroxisomes (A), lysosomes (B) and mitochondria (C,D) after stimulation with clofibrate (A-C) or phytanic acid (D).** The intensity of each pixel in the green and red channel is determined and plotted against each other. A straight line implies colocalization, whereas a cloud of points indicates no correlation between the distribution as seen in the two channels.

The relocation was also investigated for EYFP-PC-TP expressed in FBHE cells, HUVEC, HepG2 cells and NIH3T3 mouse fibroblasts after administration of 100 μM clofibrate. After stimulation and fixation of the cells, the mitochondria were stained by antibodies directed against cytochrome c oxidase subunit IV. In both FBHE cells and HUVEC, EYFP-PC-TP moved to the mitochondria (figure 8a,b). However, it can be seen that not all mitochondria show PC-TP labelling suggesting that PC-TP has relocated to a subset of mitochondria. The occurence of two populations of mitochondria is reflected in the relatively low correlaton coefficients of 0.85 for FBHE cells and of 0.65 for HUVEC. No relocation to mitochondria was observed for the HepG2 cells and NIH3T3 fibroblasts (data not shown). EYFP expressed in these cells did not relocate to mitochondria in response to clofibrate treatment. Besides clofibrate, arachidonic acid (500 μM) was also observed to induce mitochondrial relocation of EYFP-PC-TP in FBHE cells and HUVEC (figure 8c; figure 9c).

Relocation is associated with the PKC phosphorylation site at Ser^{110}

PC-TP has one putative PKC phosphorylation site at Ser^{110} (21-23). Ser^{110} is the structural homologue of Ser^{195} in steroid acute regulatory protein (StAR) (24). Upon phosphorylation StAR moves to mitochondria to deliver cholesterol to the mitochondrial membrane. Since the same mechanism might apply for PC-TP, Ser^{110} was replaced for an alanine residue by site-directed mutagenesis. After expression in *E. coli* the mutated bovine protein denoted as PC-TP(S110A) was fully active as compared to non-mutated bovine PC-TP (data not shown). Upon expression of the EYFP-PC-TP(S110A) fusion protein in FBHE cells, 100 μM clofibrate did not induce relocation to the mitochondria (figure 10a) whereas EYFP-PC-TP used as control did relocate (figure 10b). This strongly suggests that clofibrate-induced relocation to the mitochondria is associated with phosphorylation of Ser^{110}.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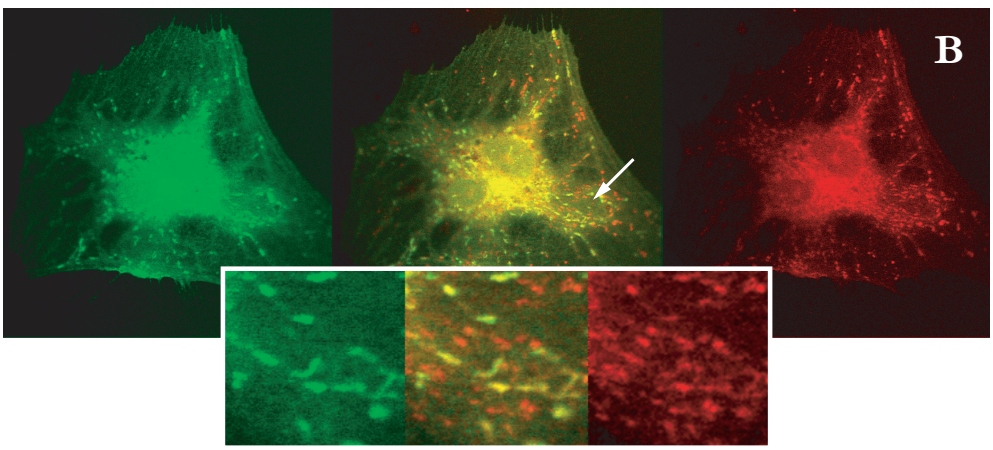
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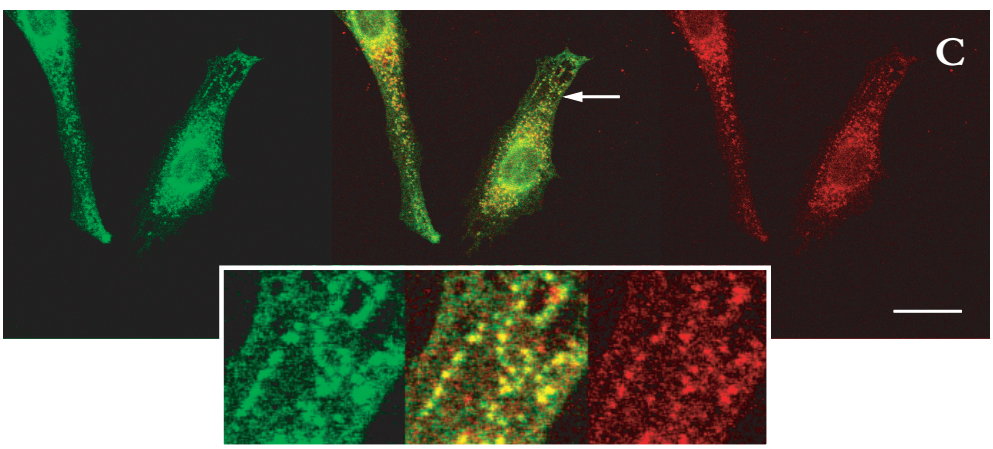
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◄Figure 8. **Mitochondrial localization of EYFP-PC-TP after stimulation with clofibrate or arachidonic acid.** Panel A: localization of PC-TP as compared to antibodies directed against TRITC-labelled cytochrome C oxidase subunit IV in FBHE cells after stimulation with 100 μM clofibrate; panel B: same in HUVEC; panel C: localization of PC-TP as compared to antibodies directed against TRITC-labelled cytochrome C oxidase subunit IV in FBHE cells after stimulation with 500 μM arachidonic acid. On the left side of each panel the green channel (PC-TP) is shown and on the right side the red channel (mitochondria). The yellow colour of the middle image indicates colocalization. Arrows indicate the position of the inset; the bar represents 20 μm of length. All images were obtained by CLSM.

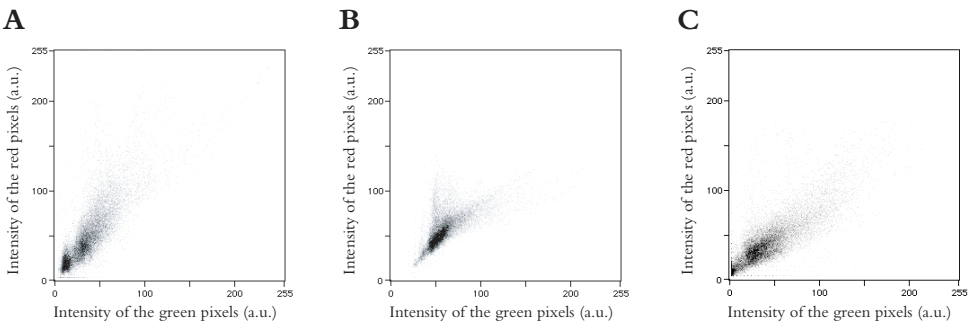

Figure 9. **Correlation plots comparing the distribution of EYFP-PC-TP with mitochondria in FBHE cells (A) and HUVEC (B) after stimulation with clofibrate and in FBHE cells after stimulation with arachidonic acid (C).** The intensity of each pixel in the green and red channel is determined and plotted against each other. A straight line implies colocalization, whereas a cloud of points indicates no correlation between the distribution as seen in the two channels.

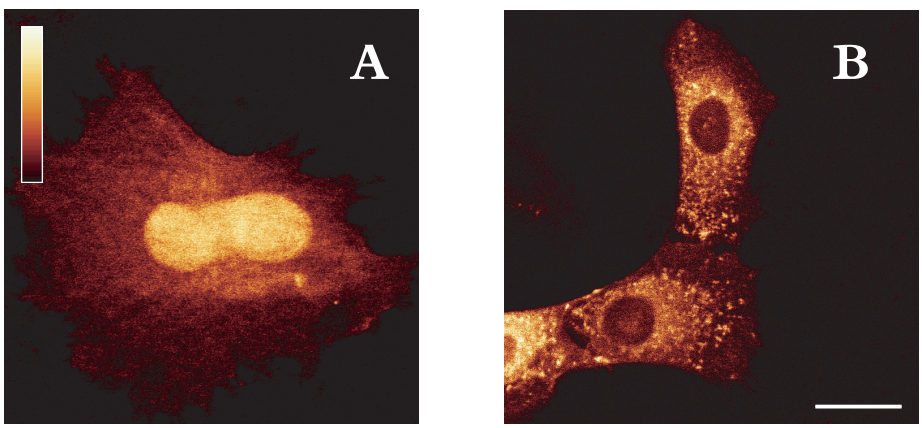

Figure 10. **Effect of the replacement of Ser^{110} with Ala on the distribution of PC-TP in FBHE cells after stimulation with clofibrate (200 μM).** Panel A: EYFP-PC-TP (S110A); panel B: EYFP-PC-TP. The bar represents a length of 20 μm. Images were obtained by CLSM.

Discussion

Despite numerous efforts, the physiological function of the highly conserved PC-TP still remains to be established (11). Here we show by confocal laser scanning microscopy that fluorescently labelled PC-TP was evenly distributed throughout the cytoplasm of FBHE cells, HUVEC, HepG2 cells and NIH 3T3 mouse fibroblasts with elevated levels in the nuclei. This latter enrichment was also observed for EYFP expression in these cells, which argues against a specific function for PC-TP in the nuclei. By fluorescence recovery after bleaching PC-TP was found to be highly mobile throughout the cell. Bleaching of PC-TP in the nuclei indicated that the mobility of PC-TP between the cytosol and the nucleus was restricted requiring a recovery period of 10 min for Oregon Green-labelled PC-TP and of 15 min for EYFP-PC-TP. Similar times of recovery have been observed after the nuclear bleaching of fluorescently labelled dextrans having the same size (57-59).

Addition of clofibrate (100 μM) to the medium of endothelial cell caused PC-TP to move to mitochondria within five min. Other peroxisome proliferators, arachidonic acid (500 μM) and phytanic acid (10 μM), could also induce this relocation. Peroxisome proliferators induce phosphorylation of serine and threonine residues by protein kinase C (PKC) (60-67). These modifications appear to be either an early event or a mechanism independent of peroxisome proliferation. When we substitute Ser^{110} that is part of a putative PKC phosphorylation site for an alanine residue, the relocation of PC-TP is abolished. This strongly suggests that the clofibrate-induced relocation of PC-TP to the mitochondria is preceded by PKC phosphorylation. The PKC isoform involved is probably specific, since rat brain PKC consisting mainly of PKCγ, was not able to phosphorylate PC-TP for more than 0.2% *in vitro* (data not shown). This can explain the absence of relocation in NIH3T3 mouse fibroblasts. Clofibrate was unable to induce the relocation of EYFP-PC-TP in HepG2 cells as well, which agrees with fibrates not being able to induce phosphorylation in these cells (64). The specific relocation of PC-TP to mitochondria upon phosphorylation strongly suggests that mitochondria contain docking proteins, which interact with the phosphorylated PC-TP. After docking PC-TP most likely stays on the outside of the mitochondria, since PC-TP does not contain a mitochondrial localization signal (68).

Clofibrate is an inhibitor of PE methyltransferase (PEMT) mediating the methylation of PE to PC, a process that takes place in the mitochondria-associated membranes (69, 70). Inhibition of PEMT impairs the lipidation of apolipoprotein B48-containing lipoproteins, leading to decreased triglyceride (TG) and

phospholipid levels in blood (71). Interestingly, clofibrate treatment did not affect blood plasma TG and phospholipid levels in PC-TP null mice (chapter 7). In view of its association with mitochondria after clofibrate stimulation, we suggest that PC-TP is a regulator of PEMT. This agrees with PC-TP not being relocated in HepG2 cells, since these cells, in contrast to their hepatic origin, display very low PEMT activity (72). On the other hand, the PE methylation pathway appears to be restricted to liver (73) whereas PC-TP is more ubiquitously expressed.

In transferring PC between membranes, PC-TP prefers PC molecular species that have a C16:0 on the *sn*-1 position and a PUFA on the *sn*-2 position (3). It is conceivable that relocation of PC-TP to mitochondria increases the amount of C16:0/PUFA-PC species in the mitochondrial membranes. The ensuing increase of membrane fluidity is reported to generate a number of responses, such as the enhancement of membrane-associated phospholipase A_2 activity (74), proton leakage (75, 76) and Ca^{2+} efflux (77). Furthermore, changes in phospholipid fatty acid composition can alter the activity of carnitine palmitoyltransferase I (78, 79) and of the pyruvate carrier (80, 81). If correct, the interaction of PC-TP with mitochondria may have a profound effect on mitochondrial function, including an increased β-oxidation. In this regard it is to be noted that fibrates exert their hypotriglyceridemic effect primarily via increased β-oxidation in the mitochondria (82).

Although many implications resulting from the relocation of PC-TP to mitochondria can be envisaged, we have as yet no direct proof that PC-TP regulates mitochondrial function. However, given that clofibrate treatment both induces relocation of PC-TP to the mitochondria and stimulates a PC-TP-mediated decrease in blood plasma TG and phospholipid levels, it is apparent that attention should be focussed on PC-TP in connection to mitochondrial lipid metabolism.

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Chapter 5

Overexpression of the phosphatidylcholine transfer protein in foetal bovine heart endothelial cells: effect on phosphatidylcholine metabolism

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Abstract

To study the role of the phosphatidylcholine transfer protein (PC-TP) in phosphatidylcholine (PC) synthesis or hydrolysis, we have used foetal bovine heart endothelial (FBHE) cells containing 2.5 times the amount of PC-TP normally found in these cells. The morphology of these cells was identical to control cells, although the growth rate differed slightly. By radiolabelling the choline metabolites by [^{14}C]choline and the phospholipids with [^{14}C]palmitic acid and [^{3}H]arachidonic acid, the participation of PC-TP in phospholipid metabolism was assessed. The following conclusions could be drawn: 1) PC-TP does not participate actively in the regulation of main flow of [^{14}C]choline in equilibrium or under relocation inducing conditions; 2) PC-TP does not influence the fatty acid composition of the different phospholipid classes in either situation. If PC-TP plays a role in PC metabolism, the effects may be local and do not influence general lipid metabolism as investigated under above conditions.

Introduction

PC-TP catalyzes specifically the intermembrane exchange and net transfer of PC *in vitro* (1, 2). Both electrostatic and hydrophobic requirements have to be met for a lipid molecule to be exchanged at the membrane interface. Minor modifications of the head group of PC reduce or even abolish transport (1). Transfer is inhibited or abolished when the distance between the phosphorus and nitrogen is decreased or increased and a methyl group on the quaternary nitrogen is removed or substituted by an ethyl or propyl group. In addition, neither 1-acyl-lysoPC (1) nor a dimeric form of PC consisting of two palmitoyl-PC molecules linked together by a disulphide bond at the methyl terminal ends of the 1-acyl chains are transported by PC-TP (3). Apart from its PC specificity, PC-TP distinguishes between PC positional isomers as well. PC-TP has the highest affinity for PC species containing a C16:0 on the *sn*-1 position and a polyunsaturated fatty acid (PUFA) on the *sn*-2 position (4)(chapter1)(chapter3).

Based on its specificity for PC, PC-TP was suggested on several occasions to play a role in PC metabolism. Concurrently, PC-TP was *in vitro* able to stimulate the cholinephosphotransferase activity (5). Cholinephosphotransferase catalyses the reaction between CDP-choline and diacylglycerol (DAG) to yield PC and CMP, which is the final step of the Kennedy or CDP-choline pathway (for a review (6)).

In addition, PC-TP was able to enhance sphingomyelin (SM) synthesis (7). PC and ceramide can be converted into SM and DAG by SM synthase (8-10). PC-TP is also thought to be involved in PC metabolism by stimulating phospholipase activity. PC-TP is highly mobile throughout the cell including the nucleus as shown by bleaching of fluorescently tagged PC-TP (chapter 4). PC-TP did not reside at specific subcellular structures, but seemed to be evenly distributed throughout the cytosol and the nucleoplasm. However, after stimulation by clofibrate, PC-TP relocates to mitochondria within 5 min. It is possible that this relocation may lead to increased levels of C16:0/PUFA-PC species in (parts of) the mitochondrial membranes so as to enhance the activity of phospholipases or to regulate phosphatidylethanolamine methyl transferase.

To study the role of PC-TP in PC synthesis or hydrolysis under conditions inducing relocation, we have used foetal bovine heart endothelial (FBHE) cells containing 2.5 times the amount of PC-TP normally found in these cells. The morphology of these cells as compared to control cells was identical, although the growth rate differed slightly. By radiolabelling the choline metabolites by [^{14}C]choline and the phospholipids by [^{14}C]palmitic acid and [^{3}H]arachidonic acid, the participation of PC-TP in phospholipid metabolism was assessed.

Experimental procedures

Materials

The pBK-CMV vector was obtained from Stratagene (La Jolla, USA). Primers were synthesised by Eurogentec (Seraing, Belgium). The FuGENE6 Transfection Reagent Kit was obtained from Roche Molecular Biochemicals (Basel, Switzerland). [*methyl*-^{3}H]choline chloride was purchased from Amersham Pharmacia Biotech AB (Uppsala, Sweden). G418, 5-bromo-4-chloro-3-indoyl-phosphate *p*-toluidine salt (BCIP), *p*-nitro blue tetrazolium chloride (NBT), Coomassie Brilliant Blue R-250 and goat-anti-rabbit IgG conjugated with alkaline phosphatase (GAR-AP) were purchased from Sigma (ST. Louis, USA). Non fat dry milk was purchased from Nutricia (Zoetermeer, The Netherlands). Silica gel 60 plates (HP-TLC plates) were obtained from E. Merck (Darmstadt, Germany). The allsphere silica column (100 x 6.4 mm; 3 μm particles) was purchased from Alltech (Breda, The Netherlands).

pBK-CMV-PC-TP construct

cDNA containing the complete coding sequence of bovine liver PC-TP was

isolated from a λMAX bovine liver cDNA library. The cDNA encoding PC-TP was cloned into the *Eco*RI site of pBluescript SK^+. Subsequently, the pBluescript-PC-TP construct was digested with *Eco*RI and *Pst*I and the ensuing insert ligated into the multiple cloning site of the pBK-CMV vector. The expression of PC-TP is regulated by the CMV immediate early promotor. The SV40 poly(A)-adenylation signal provides the signal for termination of eukaryotic transcription and polyadenylation.

Transfection

FBHE cells were grown in Dulbecco's modified Eagles medium (DMEM) containing 10% (v/v) foetal calf serum (FCS) buffered with 44 mM $NaHCO_3$ at 37°C in a 7.5% CO_2 atmosphere and 95% humidity. FBHE cells were seeded 24 h prior to transfection at 1 x 10^4 cells/cm^2. Cells were transfected with 1 μg of the pBK-CMV-bovine PC-TP construct or the empty pBK-CMV vector using the FuGENE6 Transfection Reagent Kit according to the manufacturer's instruction. After 24 h 400 μg/ml G418 was added to the medium to select the G418-resistant cells. Fresh medium containing G418 was added every 4 days and clones overexpressing PC-TP identified after three weeks of growth.

Gel electrophoresis and Western blotting

Recombinant and native PC-TP were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 12% acrylamide and 0.37% bis-acrylamide (11). Proteins were visualized by Coomassie Brilliant Blue R-250 staining or transferred to a nitrocellulose membrane (Schleicher & Schuell BA 85) by semi-dry Western blotting in a Multiphor II Nova Blot electrophoretic transfer unit (Pharmacia, Uppsala, Sweden) at 1 mA/cm^2 for 1 h at room temperature. The non-specific binding sites of the nitrocellulose membrane were blocked by incubating the membrane for 1 h in 2% non fat dry milk (w/v) in 20 mM Tris-HCl pH 7.5, 0.5 M NaCl (TBS) at 37°C. Subsequently, the blot was incubated for 1 h at 37°C with the affinity purified antibody Ab270 raised against native bovine PC-TP (affinity purified by Protein A Sepharose CL-4B) diluted 1:20 in TBS containing 0.2% non fat dry milk (w/v). The blot was washed with TBS-Tween 20 (0.05% v/v; TBS-T) (3 x 10 min) and incubated for 1 h with GAR-AP diluted 1:5000 in TBS. The blot was washed again with TBS-T (3 x 10 min). The immunoreactive proteins were visualized by incubating the blot in 10 ml 0.1 M $NaHCO_3$ pH 9.8, 10 mM $MgCl_2 \cdot 6H_2O$ containing 0.3 mg/ml BCIP and 0.15 mg/ml NBT as the colour development substrate.

[^{14}C]Choline, [^{14}C]palmitic acid and [^{3}H]arachidonic acid labelling of FBHE cells

Cells were subcultured into Ø 10 cm petri dishes in 8 ml medium and grown to a density of 1 x 10^4 cells/cm^2. Subsequently, the medium was replaced by fresh medium containing 1 μCi/ml [^{14}C]choline or 1 μCi/ml [^{14}C]palmitic acid and [^{3}H]arachidonic acid. After 32 hrs the cells were harvested (equilibrium conditions) or challenged by 100 μM clofibrate or DMSO (control) in serum-free medium for 30 min and harvested successively.

Choline metabolite and lipid analysis

Before extraction, cells were washed once with phosphate-buffered saline (PBS; 8.3 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) and harvested in PBS using a policeman. The ensuing suspension was sonicated for 15 min in a sonication bath. Subsequently, the homogenates were extracted by the method of Bligh and Dyer (12). The organic phase was dried under N_2 (g) and (phospho)lipids were separated by high performance thin layer chromatography (HP-TLC) using chloroform/methanol/water 65/25/4 (v/v/v) as mobile phase (13) ([^{14}C]choline) or by high performance liquid chromatography (HPLC) using an allsphere silica column (100 x 6.4 mm; 3 μm particles) according to Ellingson *et al.* (14) ([^{14}C]palmitic acid and [^{3}H]arachidonic acid). The aqueous phase was lyophilised and dissolved in a small volume of H_2O/ethanol, 3/1 (v/v). Aliquots of the samples were applied to a HP-TLC plate and developed in ethanol/H_2O/ammonia, 48/95/7 (v/v/v) (15). [^{3}H]Choline metabolites were quantified by scanning the HP-TLC plates with a Berthold Tracemaster 20 Automatic TLC-Linear analyser (Berthold Technologies GmbH, Bad Wildbad, Germany). [^{14}C]Palmitic acid and [^{3}H]arachidonic acid metabolites separated by HPLC were quantified by a Packard Flow Scintillation Analyzer 500 TR (Packard Instrument Company, Meriden, USA).

Results

Overexpression of PC-TP in bovine heart endothelial cells

To study the participation of PC-TP in PC metabolism, we overexpressed bovine PC-TP in FBHE cells. Therefore bovine PC-TP was cloned into the pBK-CMV vector, which is commonly used to obtain high expression levels of protein by means of its eukaryotic constitutive CMV promoter. FBHE cells were transfected with the pBK-CMV-PC-TP construct or the empty pBK-CMV vector by lipofection. Stable

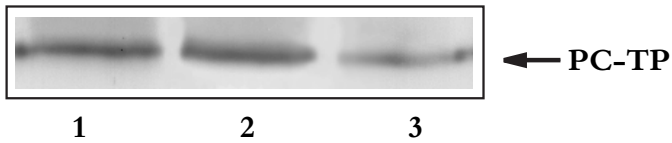

Figure 1. **Immunoblot analysis of total cell lysates from FBHE cells transfected with pBK-CMV-PC-TP.** Lane 1: 50 μg protein from OP-1 cells; lane 2: 50 μg protein from OP-2 cells; lane 3: 50 μg protein from control cells transfected with the empty pBK-CMV vector.

cell lines were selected using the antibiotic G418. Five cell lines of each transfection were selected and their PC-TP content analysed by immunoblot. Two cell lines overexpressing PC-TP (OP-1 and OP-2) and one control cell line were chosen for further experiments. The expression of PC-TP in these cell lines was quantified by densitometric scanning (figure 1). The PC-TP content in OP-1 and OP-2 was 2.7 and 2.5-fold higher than in the control, respectively. After the labelling experiments, the amount of PC-TP in OP-1, OP-2 and control cells was determined again yielding comparable levels of overexpression.

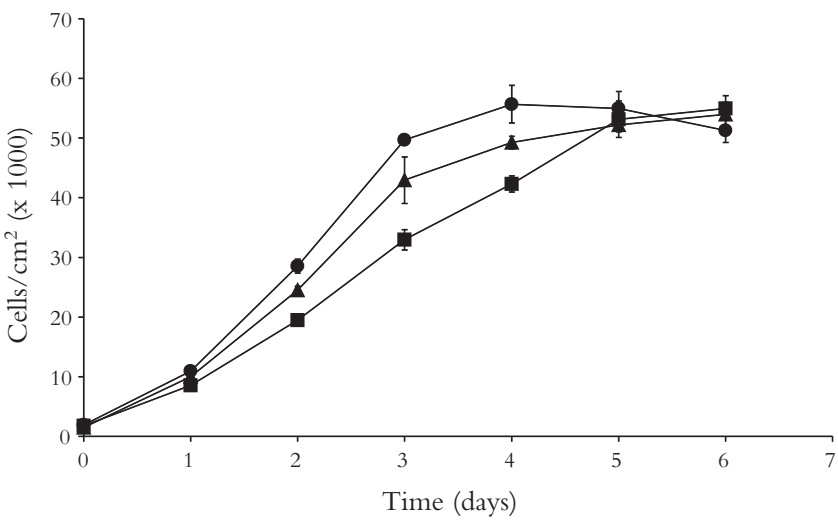

Figure 2. **Growth curves of OP-1 (squares), OP-2 (triangles) and control FBHE cells (circles).** The values are the means of three independent experiments (± S.E.M.).

Growth characteristics of OP-1, OP-2 and control cells

The growth rate of OP-1, OP-2 and control cells was determined by counting cells for 6 days (figure 2). OP-1 cells proliferated at the slowest rate, followed by OP-2 and control cells. The time needed to reach 50 % confluency differed accordingly. OP-1 cells needed 62 h, OP-2 cells 52 h and control cells 46 h to reach a density of 2.6 x 10^5 cells/cm^2. Morphological differences visible between the three cell lines were not observed.

Labelling with [^{14}C]choline

Cells were labelled with [^{14}C]choline to determine the effect of increased PC-TP levels on PC metabolism. The total radioactivity taken up by the cells was determined and used to calculate the fraction of each labelled metabolite. Overexpression of PC-TP did not induce differences in the incorporation of [^{14}C]choline in the different metabolites of OP-1 and OP-2 as compared to that of control cells after 24 h (figure 3a). Most of the radioactivity was found in PC (77%), the remainder being mainly found in SM (8%) and CDP-choline/glycerophosphocholine (8%). Clofibrate has been shown to cause massive relocation of PC-TP from the cytosol and nucleoplasm to mitochondria (chapter 4). Hence, 30 min chase experiments were performed in the presence of 100 μM clofibrate or DMSO. The [^{14}C]choline metabolite profiles were not altered by clofibrate or DMSO as compared to the equilibrium situation (figure 3b,c). The [^{14}C]choline incorporation per mg protein decreased in both chase experiments in comparison with the equilibrium situation from 8404 ± 818 cpm/mg protein (equilibrium) to 8062 ± 374 (clofibrate) and 6264 ± 883 (DMSO).

Double labelling with [^{14}C]palmitic acid and [^{3}H]arachidonic acid

Although PC-TP is a specific transporter of PC, it has a distinct preference for C16:0/PUFA-PC species (4)(chapter 1)(chapter 3). It seems likely that if PC-TP influences lipid metabolism in the cell, especially these species will be affected. We labelled OP-1, OP-2 and control cells with [^{14}C]palmitic acid and [^{3}H]arachidonic acid. Accordingly, we analysed the lipid profile of these cells after 24 h of labelling and after a 30 min chase in the presence of 100 μM clofibrate or DMSO. There was no significant difference between the three experiments (figure 4). Clearly visible is the high incorporation of arachidonic acid in phosphatidylinositol, whereas almost none ends up in SM. There is no difference in the [^{3}H]/[^{14}C] incorporation ratios of PC between the three cell lines or between the equilibrium and chase experiments. The ratio [^{3}H]arachidonic acid/[^{14}C]palmitic acid is notably in favour of the arachidonic acid, although the same amount of radioactivity was added to the cells.

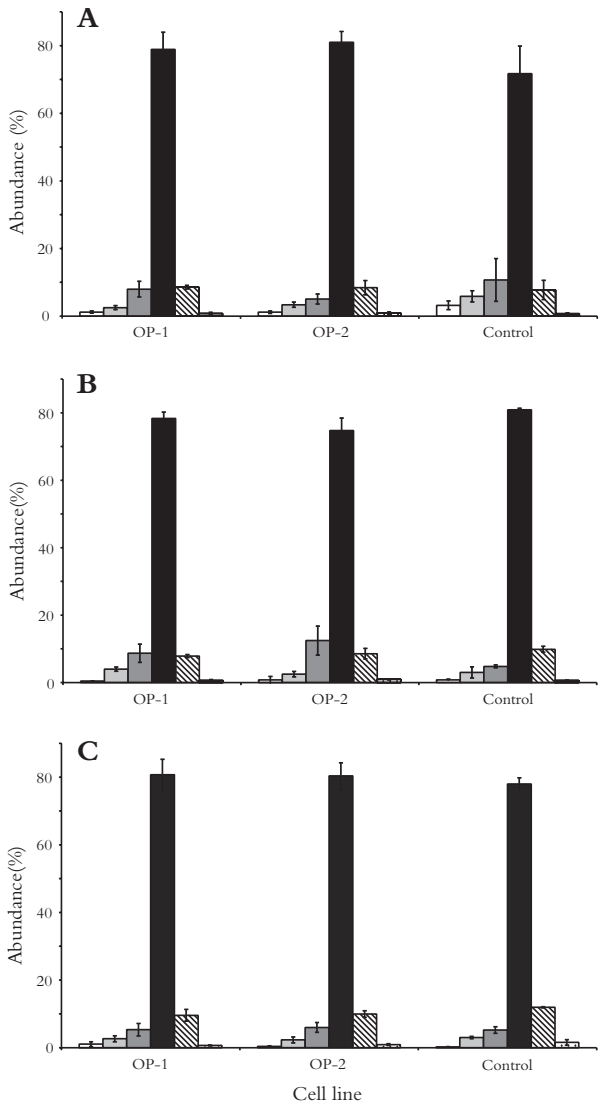

Figure 3. **Incorporation of [^{14}C]choline in its various metabolites.** [^{14}C]Choline metabolite profiles are shown after 24 h (equilibrium; A) followed by a chase of 30 min in the presence of 100 μM clofibrate (B) or DMSO (C). Presented are choline (white bars), phosphocholine (light grey bars), glycerophosphocholine and CDP-choline (dark grey bars), PC (black bars), SM (hatched bars) and lysoPC (polka dot bars). The values are the means of four (equilibrium) or three (clofibrate and DMSO) independent experiments (± S.E.M.).

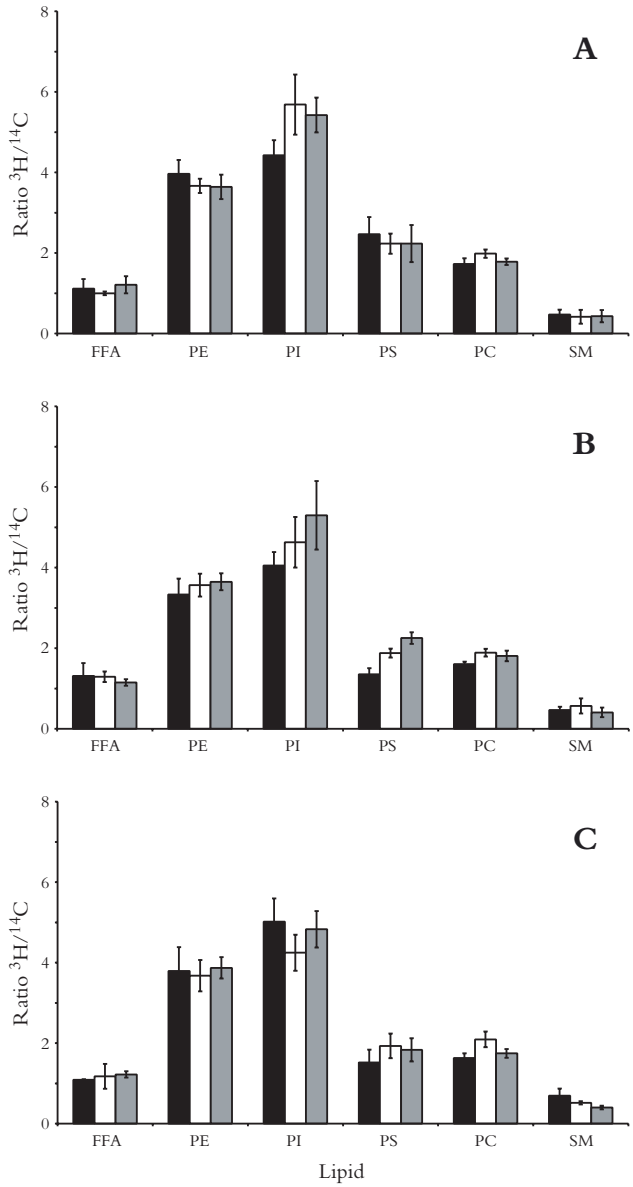

Figure 4. **The ratio [^{3}H]arachidonic acid/[^{14}C]palmitic acid incorporation in phospholipids.** Phospholipid profiles are shown after 24 h (equilibrium; A) followed by a chase of 30 min in the presence of 100 μM clofibrate (B) or DMSO (C). Presented are OP-1 (black bars), OP-2 (white bars) and control FBHE cells (grey bars). The values are the means of four (equilibrium) or three (clofibrate and DMSO) independent experiments (± S.E.M.).

The total incorporation of the [^{14}C]palmitic acid and [^{3}H]arachidonic acid per mg protein decreased during both chases from 10,505 ± 913 cpm (equilibrium) to 8,530 ± 451 (clofibrate) and 9,169 ± 593 (DMSO) and from 4,051 ± 75 cpm (equilibrium) to 3,803 ± 95 (clofibrate) and 3,818 ± 235 (DMSO), respectively.

Discussion

PC-TP is a specific transporter of PC between membranes. Because of his specificity, PC-TP was proposed to play a role in PC metabolism. PC-TP was *in vitro* able to stimulate the cholinephosphotransferase activity (5) and enhance SM synthesis (7). Here we have used overexpression of PC-TP to study the role of PC-TP in phospholipid metabolism. FBHE cells were transfected by a pBK-CMV-bovine PC-TP construct or the empty pBK-CMV vector (control). After selection by G418, two cell lines were chosen having 2.7 (OP-1) and 2.5-fold (OP-2) more protein than the control cell line. The other G418 resistant cell lines transfected with the pBK-CMV-PC-TP construct all had 1.5 to 2-fold more PC-TP than the control cell lines tested (data not shown). This indicates that it is difficult to express more than 2.5-fold the normal amount of PC-TP in FBHE cells. Overexpression of murine PC-TP in NIH3T3 mouse fibroblasts that normally contain no detectable PC-TP levels supports this observation. Although the tested transfected cell lines were G418 resistant, PC-TP could not be detected on immunoblot. However, the growth rates of the stable transfectants were, without exclusion, two-fold higher than that of control cells (data not shown). This suggests that it is difficult to express high levels of PC-TP, despite the fact that it appears that PC-TP affects cellular processes. The low levels of PC-TP after overexpression can be caused by the RLLDQ sequence found in both bovine and murine PC-TP. This motive targets cytosolic proteins for lysosomal proteolysis both upon serum deprivation of cells in culture (16-18) and in liver and heart of fasted rats (19). Murine PC-TP contains even an additional QRRDL motive.

To study the participation of PC-TP in PC metabolism, cells were labelled with [^{14}C]choline. In equilibrium, there was no difference in [^{14}C]choline incorporation in its various metabolites between the two PC-TP overexpressing cell lines and the control cell line. Furthermore, after a chase in the presence of clofibrate or DMSO, still no change in metabolite profile was observed. The cells were metabolically active, since the total amount of radioactivity present in the cells decreased during the chase. If PC-TP has any effect on the incorporation in or the release of [^{14}C]choline from

PC, the effect is minor and not visible under these conditions. This can be caused by increased proteolysis of PC-TP in lysosomes as a result of the absence of serum during the chase experiments, although the degradation half-time of this pathway is in general more than 40 h (17). Taken together, this would suggest that PC-TP is not involved in regulating the main choline flow in the cell.

PC-TP transfers C16:0/PUFA-PC species faster than other PC species (4)(chapter 3). This could indicate that PC-TP is involved in cellular processes related to specific fatty acids, such as the release of arachidonic acid by phospholipase A_2. Therefore, we have determined the [^{14}C]palmitic acid/[^{3}H]arachidonic acid incorporation and hydrolysis ratio under equilibrium conditions and after a chase in the presence of clofibrate and DMSO. There were no indications in either experiment that higher levels of PC-TP enhanced the release of arachidonic or palmitic acid.

In conclusion, it seems that *in vivo* PC-TP does not participate actively in the regulation of main flow of [^{14}C]choline in equilibrium or under relocation inducing conditions. In addition, PC-TP does not influence the fatty acid composition of the different phospholipid classes in either situation. If PC-TP plays a role in PC metabolism, the effects may be local and do not influence general lipid metabolism as investigated under these conditions. If this is the case, it could be worthwhile to isolate mitochondria and associated membranes to determine the effect of PC-TP on these membranes.

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Chapter 6

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

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Abstract

Pc-tp null mice were generated to study the a number of proposed functions for the phosphatidylcholine transfer protein (PC-TP), such as a role in bile formation, lung surfactant production and the facilitation of enzymatic reactions involving phosphatidylcholine (PC) synthesis or breakdown. Remarkably, these mice were normal and had 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 was normal. That PC-TP does not participate in biliary lipid secretion is in agreement with our finding that PC-TP levels decreased >10-fold around 2 weeks after birth, when bile formation starts. Whereas PC-TP is abundant in the liver of mouse pups, 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.

Introduction

PC-TP mediates specifically the intermembrane exchange and net transfer of PC (1, 2). Even minor alterations of the phosphorylcholine head group greatly diminish or eliminate transfer. PC-TP is predominantly expressed in tissues exhibiting a high lipid turnover such as liver, kidney and intestine. On the basis of its tissue distribution and binding specificity, a variety of functions can be proposed. In chapter 5 we have investigated a possible role for PC-TP in PC synthesis and hydrolysis. PC metabolites in normal foetal bovine heart endothelial (FBHE) cells and FBHE cells containing 2.5-fold the PC-TP levels normally found in these cells were radiolabelled by [^{14}C]choline and [^{14}C]palmitic acid and [^{3}H]arachidonic acid. PC-TP did not participate actively in the regulation of main flow of [^{14}C]choline or in the remodelling of C16:0 and C20:4 attached to PC.

On several occasions, PC-TP has also been implied to be involved in secretion of PC. In liver, PC-TP is thought to deliver PC to the PC-translocator, Mdr2 P-glycoprotein (P-gp), in the bile canalicular membrane (3-9). PC-TP obtains its substrate PC at the place of PC synthesis, the cytosolic leaflet of the endoplasmic reticulum and the Golgi apparatus (10), and shuttles 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 (3). In lung, PC-TP is proposed

to be involved in the PC transfer between the endoplasmic reticulum and the surfactant containing lamellar bodies. Surfactant is produced by the alveolar type II cells, which are highly enriched in phospholipid transfer activity as compared to the whole lung (11). In addition, PC-TP levels were maximal in the foetal mouse and rat lung in the period of accelerated surfactant formation (12, 13).

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.

Experimental procedures

Materials

The kit used for rapid amplification of cDNA ends (5'RACE) and ESGRO-leukemia inhibitory factor was purchased from Gibco BRL (Breda, The Netherlands). A23187 calcium ionophore, G418 and endotoxin from *Escherichia coli* serotype O111B4 was obtained from Sigma (St. Louis, USA). The enhanced chemiluminescence kit a Resource S was obtained from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Silica-60 TLC plates were purchased from E. Merck (Darmstadt, Germany). Imaging screens was obtained from Fuji (Rotterdam, The Netherlands).

Cell culture

Embryonic stem cells, clone E14, were grown on mitotically inactivated mouse embryonic fibroblasts in Glasgow modified Eagles medium (MEM) supplemented with 10% foetal calf serum (FCS), 1x nonessential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM β-mercaptoethanol, 10^3 units ESGRO- leukemia inhibitory factor/ml. 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 (14).

Isolation of mouse and human cDNAs

Two independent 5'RACE reactions were performed on 1 μg mouse RNA according to the manufacturer's instructions to complete the 59 sequence information lacking from the mouse PC-TP clone isolated by Geijtenbeek *et al.* (13). Both fragments were sequenced, and missing coding sequence was ligated to the 39 part of the mouse cDNA. The human cDNA was sequenced from the clone

aa030013 obtained from the I.M.A.G.E. Consortium library (15). 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).

Disruption of the *Pc-tp* gene by homologous recombination

From a lambda EMBL3 genomic 129/Ola DNA library (16) 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 figure 2a. 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 the pBS-KS vector. Subsequently the 39 arm, a 1.9-kb XbaI 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 x 10^7 embryonic stem cell line (E14 ES) with a BioRad GenePulser at 3 mF 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 39 and the 59 *Pc-tp* probe. Of 650 G418-resistant clones, three were identified as positives giving the expected wild-type bands of 5 kb (39 probe) and 10 kb (59 probe) and the mutated fragments of 12 kb (39 and 59 probe).

Protein analysis

Tissue was homogenized in 10 mM Tris-HCl pH7.35, 0.25 M sucrose, 1 mM EDTA, 1 mM PMSF, 1 mg/ml leupeptin, 1 mg/ml pepstatin, 1 mg/ml aprotinin. Subsequently, cells were disrupted by 20 strokes through a 23-G needle. Nuclei, mitochondria, and membranes were removed by centrifugation with 1,000 x *g*, 10,000 x *g* and 100,000 x *g*, respectively. Protein concentration was determined by using a Bio-Rad protein assay. Cytosolic proteins were separated in a 12.5% sodium dodecyl sulphate-polyacrylamide 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. The phosphatidylinositol transfer proteins were detected by the polyclonal antibody Ab9026 recognizing both the PI-TPα and the PI-TPβ form (17), and the non-specific lipid transfer protein (nsL-TP) was detected

by the polyclonal antibody Ab8723.

PC-TP transfer activity

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 column 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 (18). The donor vesicles consisted of 2-pyrenyl-decanoyl-PC/eggPC/phosphatidic acid (PA)/trinitrophenol-PE (10:70:10:10 mol%) and the acceptor vesicles of egg PC and PA (95:5 mol%). PI-TPα, PI-TPβ, 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/eggPC/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 (19). Cholesterol was assayed enzymatically with cholesterol oxidase (20). Total bile salt concentration was measured spectrophotometrically with 3-αhydroxysteroid dehydrogenase (21). 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 (22).

Bronchoalveolar lavage

Mice were lightly anesthetized by inhalation of isoflurane, and endotoxin from *E. coli* serotype O111B4 in 50 ml isotonic saline was inoculated intranasally. Control mice were inoculated intranasally with 50 ml of isotonic saline only. At 6 h 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 x *g* for 5 min at 4°C, and supernatants were stored at -80°C until lipid analysis.

Labelling and separation of lipids

Cells (1 x 10^6) were preincubated in Tyrode's salt solution for 1 hr at 37°C to reduce cellular choline and labelled with [^{14}C]choline chloride (1 mCi/ml medium) or with [^{14}C]ethanolamine (0.2 mCi/ml medium) at 37°C. Lipids were extracted according to Bligh and Dyer (23). The organic phase was dried under N_2 and the lipids applied to silica-60 TLC plates 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 phase, the samples were dried in a Speed-Vac (Savant, Hicksville, USA), 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 and the radiolabelled spots quantified. Identification of PC molecular species was performed by HPLC (24) and on-line electrospray mass spectrometry (25).

Isolation and incubation of bone marrow-derived mast cells (BMMCs)

Three independent samples of BMMCs were prepared and cultured as described (26). 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 with 0.2 mM A23187 calcium ionophore at 37°C (27). 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 (28). For lipid analysis and labelling studies, the reaction was stopped by Bligh and Dyer extraction (23).

Results

Generation and characterization of *Pc-tp* knockout mice

The murine genome contains only a single gene for PC-TP since low-stringency hybridization of mouse genomic Southern blots with *Pc-tp* cDNA revealed no

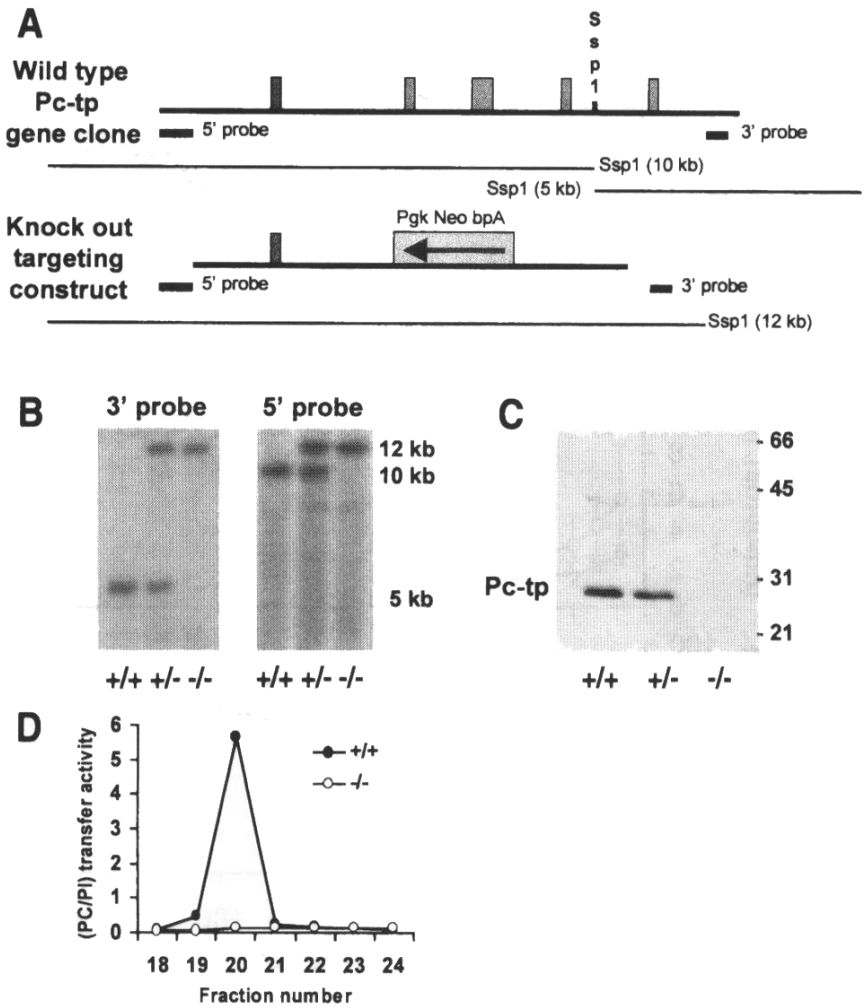

Figure 1. **Targeting of the *Pc-tp* gene by homologous recombination.** (A) Structure of the five *Pc-tp* exons in the isolated genomic clone and the targeting vector with the Neomycin cassette flanked by 4.9 kb and 1.9 kb of 59 and 39 arms, respectively. The locations of the 59 and the 39 probes flanking 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 59 or the 39 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 *Experimental Procedures* 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 mg proteinylane). (D) PC-TP activity in pup liver cytosol fractions from the Resource S column. PC and phosphatidylinositol (PI) transfer activity were determined as in *Experimental Procedures*. 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.

additional band (data not shown). In addition, no homologue of PC-TP could be identified by searching GenBank and by BLAST (29). Chimeric mice were obtained by injection of two independently isolated *Pc-tp* (+/-) embryonic stem cell clones (T5 and M8) into blastocysts following standard procedures (30). 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 months of age ($n = 6$). Extensive histological analysis of 6-month-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 knockout mice raised the question whether the gene disruption had been successful. Southern blot analysis with 59 and 39 probes resulted in the expected genomic fragments (figure 1b). The 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 (figure 1c). 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 α and β (PI-TP α/β) 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). PC transfer activity resulting from PC-TP was absent in the *Pc-tp* (-/-) pup liver (figure 1d).

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 (3). 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* knockout 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 h from 2-month-old male mice by cannulating the gallbladder after distal ligation of the common bile duct. Compared with wild-type mice [2.9 ± 0.6 μmol/ 2 h per 100 g (n = 2)], secretion of choline containing phospholipids into bile was not diminished in *Pc-tp* (+/-) [3.0 ± 0.1 μmol/2 h per 100 g (n = 2)] or *Pc-tp* (-/-) mice [3.0 ± 0.5 μmol/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

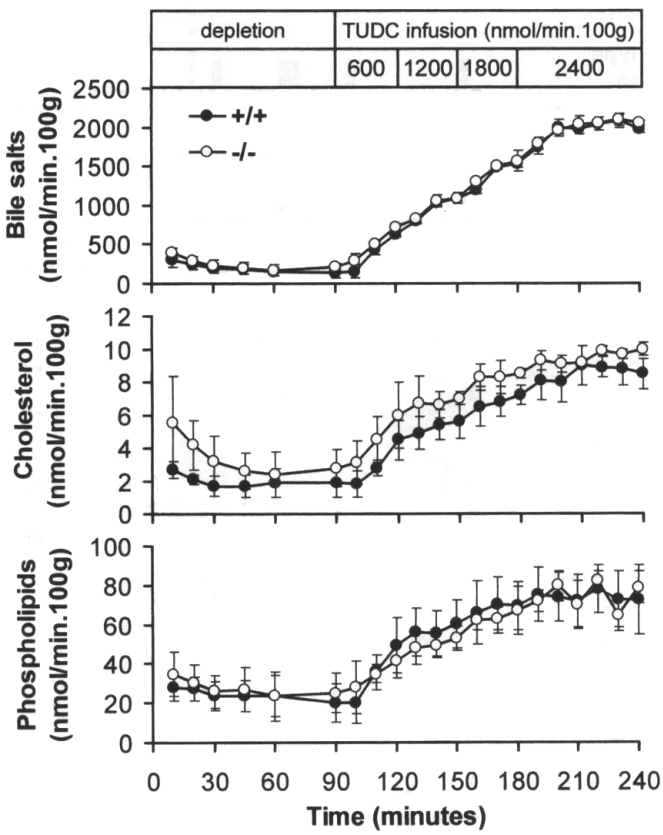

Figure 2. **Analysis of bile from *Pc-tp* (+/+) and (-/-) mouse strains during i.v. infusion of TUDC.** Wild-type (F) and *Pc-tp* (-/-) mice (E) were cannulated in the gallbladder; bile was sampled for 90 min to deplete the bile salt pool and then TUDC was infused via a tail vein at the indicated increasing rates. Data represent means of three mice (± SD). Bile salt secretion is shown on top, cholesterol secretion in the middle and phospholipid secretion at the bottom.

Table 1. **Molecular species composition of PC present in bile and lung surfactant.**

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

increased PC secretion, just like wild-type mice (figure 2). 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 (data not shown). PC-TP has the highest affinity for PC with a fatty acid composition resembling that of bile PC (31)(chapter 1)(chapter 3). This led to the suggestion that PC-TP determines the selection of the PC species characteristic for bile (8). However, the fatty acids in bile PC of *Pc-tp* (-/-) mice are indistinguishable from those of wild-type mice (table 1a).

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. 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

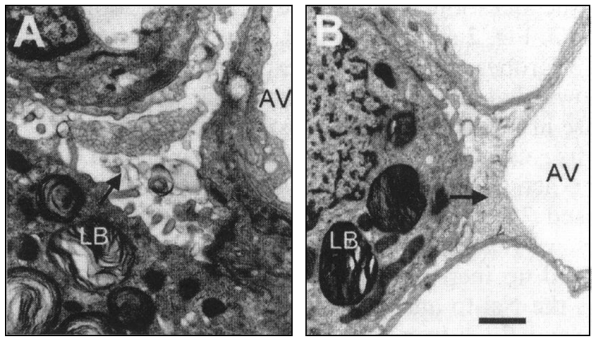

Figure 3. **Electron microscopic analysis of type II cells of *Pc-tp* (+/+) lung (A) and *Pc-tp* (-/-) lung (B).** LB = lamellar bodies; AV = alveolar spaces; arrows point to surfactant secreted from the type II cells. Bar represents 1 μm.

either. Lamellar bodies look typical, and surfactant is secreted normally (figure 3). 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 1b).

Table 2. **Tissue distribution of mouse PC-TP determined with an antibody against PC-TP**. Total protein was isolated from the different tissues and analyzed by immunoblotting with the polyclonal antibody Ab6221. The relative expression levels are indicated by - (not detectable) to +++++ (highest expression).

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	+++++

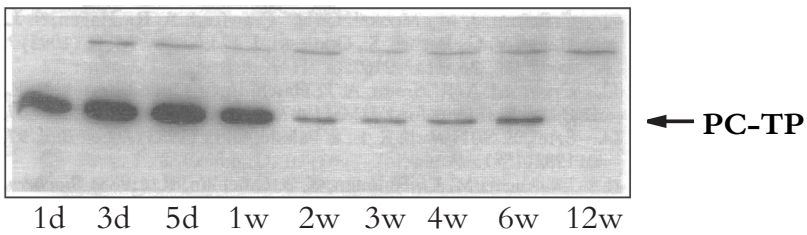

Figure 4. **PC-TP levels in mouse liver as a function of time after birth.** Equal amounts of protein from liver cytosol isolated from *Pc-tp* (+/+) mice of different age were loaded on the gel. PC-TP expression was detected by incubating the Western blot with the polyclonal antibody Ab6221 and visualized with the enhanced chemiluminescence procedure as described in *Experimental Procedures.*

Tissue distribution and cellular 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-month-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 rat PC-TP levels (12) and mRNA PC-TP levels in mouse (13)(chapter 1). 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 (figure 1c). PC-TP is high in the first week after birth but falls to a hardly detectable level within 2 weeks after birth (figure 4). Immunohistochemical analysis showed, however, that PC-TP is present in hepatocytes of 8-week-old mice (figure 5c). 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 (figure 5e) and absent from mature spermatozoa. The subcellular localization of PC-TP as determined by these light microscopical pictures is compatible with the nuclear/cytosolic localization of PC-TP under non-stimulatory conditions.

PC metabolism

To test whether the absence of PC-TP has an effect on the synthesis of PC, we labelled 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 weeks to get 98% pure primary cells. We found no effect of the presence of PC-TP on the labelling kinetics in these cells (figure 6). Also the total phospholipid composition under steady-state conditions and the different PC species did not differ significantly between *Pc-tp* (+/+) and (-/-) cells (data not shown).

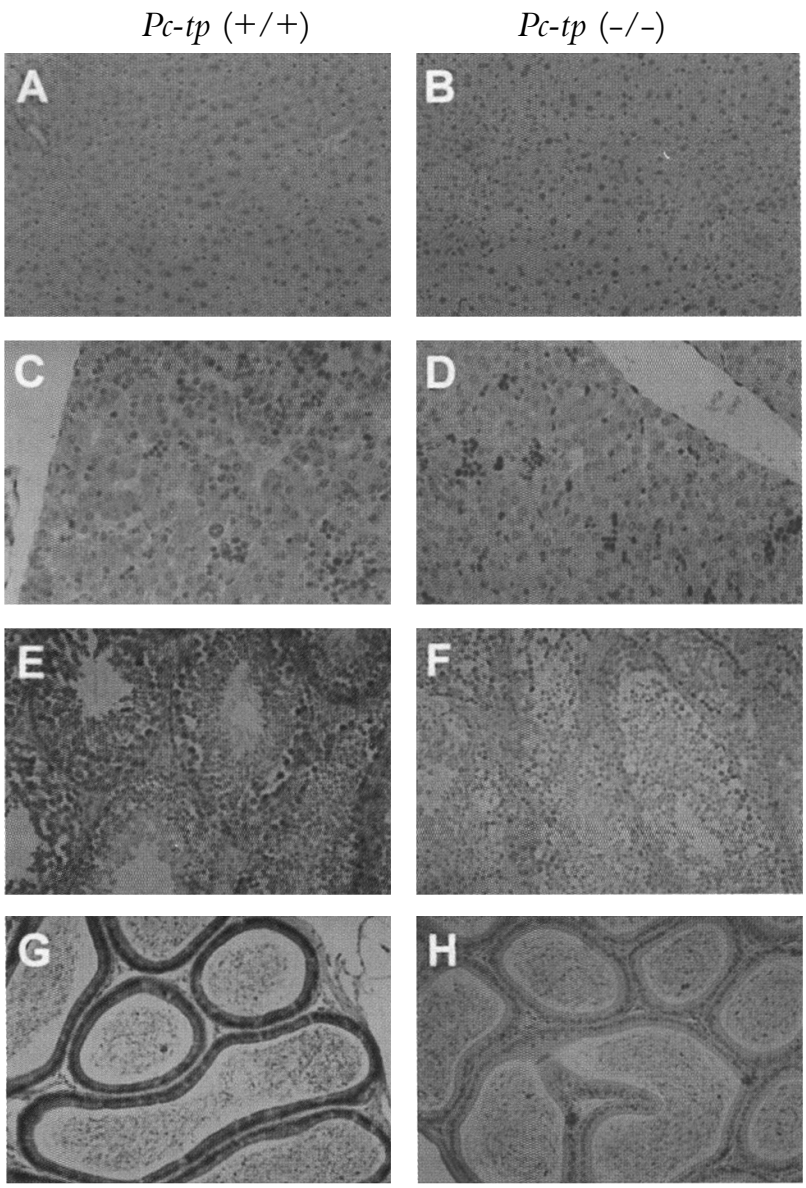

Figure 5. **Immunohistochemical detection of PC-TP in sections of formaldehyde fixed paraffin sections.** Sections were incubated with a 1:50 dilution of the polyclonal antibody Ab6221 for A–D and Ab6222 for E–H as before (36). (A) Section of an 8-week-old *Pc-tp* (+/+) liver; (B) section of an 8-week-old *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-week-old *Pc-tp* (+/+) testis; (F) section of an 8-week-old *Pc-tp* (-/-) testis; (G) section of an 8-week-old *Pc-tp* (+/+) epididymis; and (H) section of an 8-week-old *Pc-tp* (-/-) epididymis.

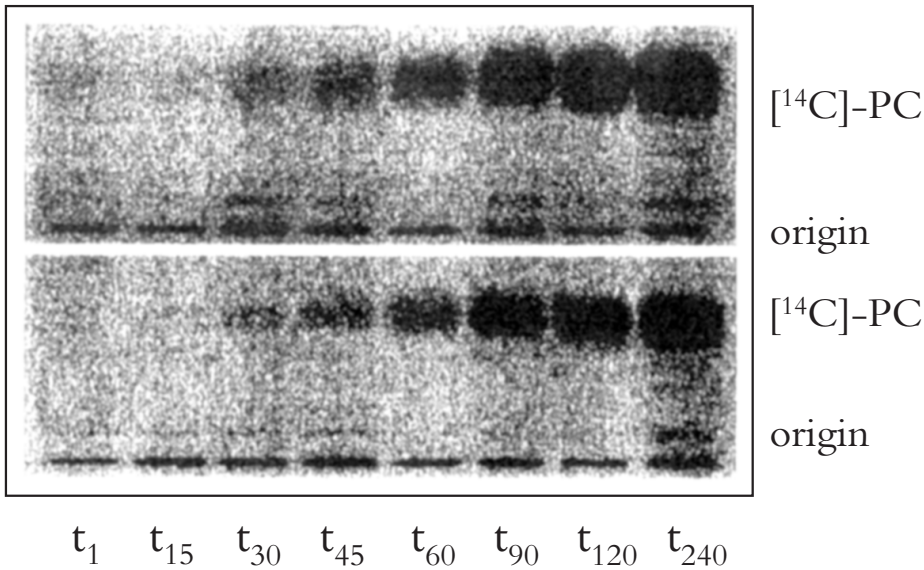

Figure 6. **Labelling kinetics of PC in BMMCs.** Shown is the [^{14}C]choline incorporation in PC during 240 min in wild type (upper panel) and *Pc-tp* (-/-) BMMCs (lower panel).

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 Ca^{2+} sensitive phospholipase A_2 (PLA_2). On stimulation of this enzyme with Ca^{2+}, arachidonic acid is liberated, which can be converted into the leukotrienes that are released from the BMMCs (27). Three independent wild-type and three independent *Pc-tp* (-/-) BMMC cell cultures were labelled to equilibrium with [^{14}C]choline for 72 h. We then stimulated PC hydrolysis with the Ca^{2+} ionophore A23187 and analyzed the formation of lysoPC and the synthesis and secretion of leukotrienes. The absence of PC-TP had no effect on PC hydrolysis (figure 7) or on leukotriene synthesis. In both *Pc-tp* (+/+) and *Pc-tp* (-/-) BMMCs, we observed a clear increase of dihydroxyeicosatetraenoic acids (DiHETEs) and leukotriene-B4s (LTB4s) after stimulation. The amounts of LTB4 ($105 \pm 28/10^7$ 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 ± 42 and $17 \pm 4/10^7$ cells, respectively).

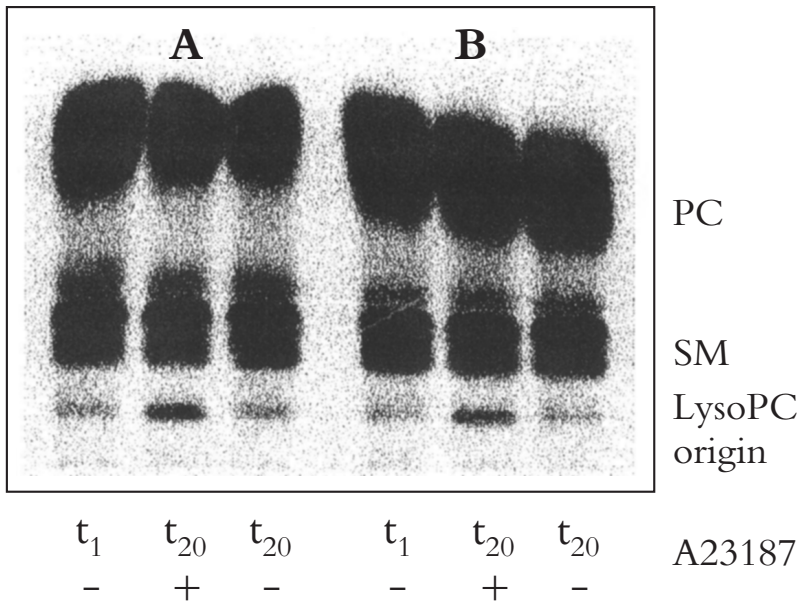

Figure 7. **PC hydrolysis in BMMCs.** Three independent wild-type (panel A) and three independent *Pc-tp* (-/-) BMMC cell cultures (panel B) were labelled to equilibrium with [^{14}C]choline for 72 h. PC hydrolysis was stimulated with the Ca^{2+} ionophore A23187.

GPC formation

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 (32-35). GPC is made from precursor PC by removing the two fatty acids (36). 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 (for review: (37)). 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.

Discussion

Previous work has led to the idea that PC-TP plays an essential role in providing PC for secretion into the bile. The results presented here refute this hypothesis. Maximal rates of PC secretion into bile are unaltered in our *Pc-tp* (-/-) mice. It is unlikely 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 α/β. 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. A putative role for PC-TP in lung surfactant production is also highly unlikely. *Pc-tp* null mice have normal surfactant production and no deviations in morphology of the alveolar type II cells. In conclusion, PC-TP is not involved in the secretion of PC by hepatocytes or alveolar type II cells.

High levels of PC-TP are found in BMMCs of wild type mice. Therefore these cells were chosen to study the effect of the absence of PC-TP on PC metabolism in the cells. We found no effect of the presence of PC-TP on the labelling kinetics or the total phospholipid composition under steady-state conditions and the different PC species. Stimulation of PC hydrolysis led to the generation of lysoPC in both wild type and *Pc-tp* (-/-) BMMCs. Furthermore, leukotriene synthesis and secretion is not impaired in *Pc-tp* (-/-) BMMCs. These results obtained using knockout cells confirm the results in chapter 5 where no effect on PC metabolism in cells overexpressing PC-TP was found; not even under relocation-inducing conditions. This, together with the fact that the localization of PC-TP is not influenced by a large variety of PC metabolism affecting stimuli, makes it very unlikely that PC-TP plays a role in PC synthesis, hydrolysis or sphingomyelin synthesis. In agreement with this, no differences in labelling kinetics or phospholipid composition were found in radiolabelled homogenized pup livers (results not shown).

PC-TP is expressed highly in the epithelial cells covering the sperm ducts in the epididymis. These cells secrete high amounts of glycerophosphocholine (GPC), which is a epididymal marker in human fertility (33, 34). Since GPC is made by PC hydrolysis (36), a role for PC-TP in the synthesis of GPC was considered. However, the fertility of the male *Pc-tp* (-/-) mice was not altered, nor was the GPC content of the epididymis. GPC also regulates the osmolarity of the epithelial cells facing the

distal part of the tubuli in kidneys (for review: (37)). Kidneys also express relatively high levels of PC-TP (table2)(chapter 1). However, no morphological abnormalities were found by lightmicroscopical analysis of *Pc-tp* (-/-) kidneys. In conclusion, PC-TP does not contribute to the production of GPC, or compensation occurs when this protein is lacking.

The analysis of *Pc-tp* (-/-) mice appears to eliminate functions previously proposed and additional functions that could follow from its transport specificity and tissue distribution. Although these mice do not display a distinct phenotype, there are still possibilities to challenge these mice. PC-TP relocates to mitochondria upon clofibrate addition. If this relocation is related to mitochondrial function, severe effects can be expected if these mice are challenged by a clofibrate-containing diet. Other diets, such as a low fat diet or a low choline diet could also provide evidence for the function of PC-TP.

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Chapter 7

Peroxisome proliferators are unable to lower plasma lipid levels in *Pc-tp* null mice

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Abstract

Our previous analysis of *Pc-tp* null mice indicated that the phosphatidylcholine transfer protein (PC-TP) is not essential for normal lipid metabolism in these mice (chapter 6). We have now found, however, that the body weight of the *Pc-tp* (-/-) mice was approximately 15% higher ($p < 0.05$) than that of *Pc-tp* (-/-) mice after six months on a regular diet. Despite this increased body weight, the *Pc-tp* (-/-) mice did not show morphological signs of obesity. In search of an explanation, we challenged the (-/-) and (+/+) mice with diets containing the PPARα activators clofibrate (0.5% w/w) and phytol (0.5% w/w) for a period of three weeks. During this period, the *Pc-tp* null and *Pc-tp* (+/+) mice showed no difference in body weight development, despite the increased food intake of the *Pc-tp* (+/+) mice, especially those on clofibrate-enriched diets. This suggests that the *Pc-tp* (+/+) mice have a higher metabolic rate than the (-/-) mice. Furthermore, clofibrate significantly ($p < 0.05$) reduced total triglyceride and phospholipid levels in blood plasma of *Pc-tp* (+/+) mice but not of *Pc-tp* (-/-) mice. Phytol reduced plasma lipid levels as well, but less prominently as clofibrate. The lipid levels in the liver of the two groups of mice were similar for each diet. Yet, the PC-TP protein levels in the liver of *Pc-tp* (+/+) mice increased 3.5-fold in case of the phytol diet and 7.0-fold in case of the clofibrate diet. Based on these data we postulate that increased levels of PC-TP are linked to an enhanced plasma triglyceride and phospholipid metabolism, thereby affecting body weight.

Introduction

PC-TP mediates *in vitro* specifically the intermembrane exchange and net transfer of phosphatidylcholine (PC)(1, 2). Apart from its PC specificity, PC-TP distinguishes between PC molecular species with the highest affinity for a palmitic acid on the *sn*-1 and a polyunsaturated fatty acid (PUFA) on the *sn*-2 position (3)(chapter 1)(chapter 3). PC-TP is mainly expressed in tissues exhibiting high lipid turnover such as liver, kidney and intestine (4-7).

Based on PC specificity, PC species preference and tissue distribution, a number of functions for PC-TP can be postulated such as the supply of PC required for bile formation (8-10) or lung surfactant production (4) and the facilitation of enzymatic reactions involving PC metabolism (5, 11, 12). However, *Pc-tp* null mice show no defects in these proposed functions (chapter 6). Even though these mice do not

display a phenotype, evolutionary redundancy is unlikely given that PC-TP is highly conserved in mammals and has not been identified in *Saccharomyces cerevisiae*, *Caenorhabditis elegans* or *Drosophila melanogaster* as determined by BLAST (13).

The transcription of many genes involved in (phospho)lipid transport and metabolism is regulated by the peroxisome proliferator-activated receptor α (PPARα) (14, 15). In line with this, the promoter sequence PC-TP contains a PPAR/retinoic X receptor (RXR) heterodimer binding-site (16). Its tissue-specific expression and its possible regulation by PPARα activators could indicate a more general role for PC-TP in lipid homeostasis rather than the regulation of PC secretion, *de novo* PC synthesis or eicosanoid production. Since clofibrate and phytol are well-known PPARα activators (17-20), we have challenged wild type and *Pc-tp* null mice with clofibrate- and phytol-containing diets. Here we show that PC-TP is upregulated by these activators. In addition, we have determined the triglyceride (TG), cholesterol and phospholipid levels in blood plasma and liver of *Pc-tp* (-/-) and *Pc-tp* (+/+) mice as well as the PC plasma molecular species composition.

Experimental procedures

Materials

Antibodies raised against rat liver acyl-CoA oxidase were kindly provided by Prof. Dr. H.F. Tabak (Department of Biochemistry, Academical Medical Centre, Amsterdam, The Netherlands). Clofibrate, phytol, 5-bromo-4-chloro-3-indoyl-phosphate *p*-toluidine salt (BCIP), *p*-nitro blue tetrazolium chloride (NBT), Coomassie Brilliant Blue R-250, goat-anti-rabbit IgG conjugated with alkaline phosphatase were purchased from Sigma (ST. Louis, USA). Standard rodent chow was purchased from Altrumin (Hannover, Germany). Non fat dry milk was purchased from Nutricia (Zoetermeer, The Netherlands). Silica gel 60 plates were obtained from E. Merck (Darmstadt, Germany).

Dietary intervention studies

All mice used had a 50% FVB and 50% Ola/129 genetic background and have been described in chapter 6. The average age at the start of the experiment was 6 months and the average bodyweight ± S.E.M was for the *Pc-tp* (-/-) mice 39.1 ± 0.9 g and for the *Pc-tp* (+/+) mice 33.7 ± 0.7 g. During the experiment the mice were placed in separate metabolic cages and their food consumption and bodyweight were determined three times a week. The first seven days the mice were kept on normal

chow until their bodyweight had stabilised; an initial body weight loss of approximately 10% was observed for all mice. Then they were given normal, phytol-enriched or clofibrate-enriched chow. These diets were prepared by adding 5 mg phytol or 5 mg clofibrate per gram of chow. These chows were kindly provided by Dr. U. Seedorf (Westfälische Wilhelms-universität Münster, Germany) and before use sterilized by Gammaster (Ede, The Netherlands). At day 21 the animals were anaesthetized with isofurane and blood plasma was collected with heparin. Then they were euthanized by cervical dislocation and the organs were taken and directly frozen for further analysis. Half the liver was directly fixed with formaldehyde, and was used for histology.

Gel electrophoresis and Western blotting

Mouse liver (40 mg) was homogenised in 10 mM Tris-HCl pH 7.4, 0.25 M sucrose, 1 mM EDTA, using a potter type 853202 (B. Braun Melsungen AG, Melsungen, Germany) at 1000 rpm/min. The ensuing homogenate was centrifuged for 15 min at 17,500 x *g* at 4°C. The proteins in the supernatant (liver cytosolic fraction) were separated by SDS-polyacrylamide gel electrophoresis in 12% acrylamide and 0.37% bis-acrylamide (21) and transferred to a nitrocellulose membrane (Schleicher & Schuell BA 85) by semi-dry Western blotting in a Multiphor II Nova Blot electrophoretic transfer unit (Pharmacia, Uppsala, Sweden) at 1 mA/cm^2 for 1 h at room temperature. The non-specific binding sites of the nitrocellulose membrane were blocked by incubating the membrane for 1 h in 2% non fat dry milk (w/v) in 20 mM Tris-HCl pH 7.5, 0.5 M NaCl (TBS) at 37°C. Subsequently, the blot was incubated for 1 h at 37 °C with the affinity purified antibody Ab6221 raised against truncated recombinant mouse PC-TP (affinity purified by Protein A Sepharose CL-4B) diluted 1:20 in TBS containing 0.2% non-fat dry milk (w/v). The blot was washed with TBS-Tween 20 (0.05% v/v; TBS-T) (3 x 10 min) and incubated for 1 h with goat-anti-rabbit IgG conjugated with alkaline phosphatase diluted 1:5000 in TBS. The blot was washed again with TBS-T (3 x 10 min). The immunoreactive proteins were visualized by incubating the blot in 10 ml 0.1 M $NaHCO_3$ pH 9.8, 10 mM $MgCl_2 \cdot 6H_2O$ containing 0.3 mg/ml BCIP and 0.15 mg/ml NBT as the colour development substrate.

Lipid analysis

Lipids were extracted from blood plasma and liver by the method of Bligh and Dyer (22). Triglyceride and cholesterol levels were analyzed by an established enzymatic method (Roche, Basel, Switzerland) on the Cobas Mira S autoanalyzer.

Phospholipids were separated by high performance thin layer chromatography (HP-TLC) using chloroform/methanol/water 65/25/4 (v/v/v) as mobile phase (23). Phospholipid classes were identified by comparing the spots to standards using iodine vapour. The phosphorus content of the individual spots was determined according to Rouser *et al.* (24). PC, lysoPC, SM and PE molecular species were quantified by mass spectrometry (25).

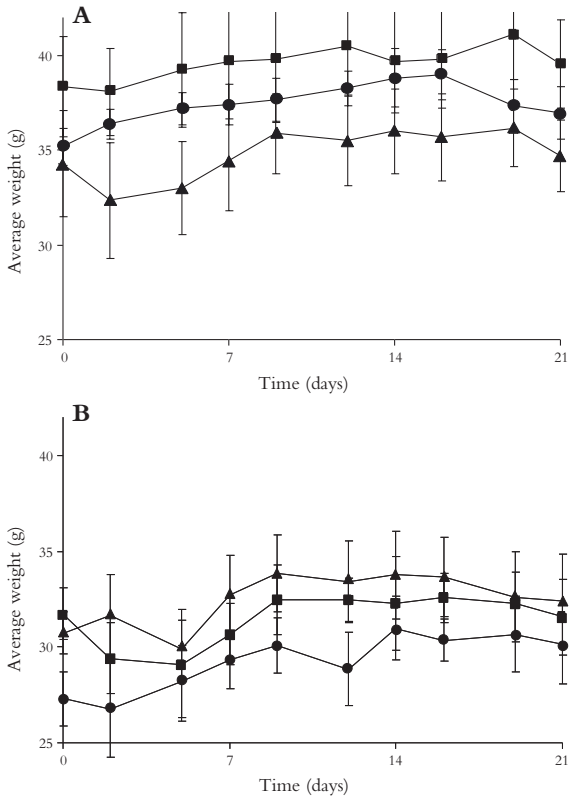

Figure 1. **Average body weight development of mice receiving control (squares), phytol enriched (triangles) or clofibrate enriched diets (circles).** Panel A: *Pc-tp* (-/-) mice. Panel B: *Pc-tp* (+/+) mice. Data are the mean of five mice (± S.E.M).

Results

Body weight of *Pc-tp* (-/-) and *Pc-tp* (+/+) mice

At the onset of the dietary intervention studies, the body weight of the six months old *Pc-tp* null mice was 15% higher ($p < 0.05$) than that of *Pc-tp* (+/+) mice although

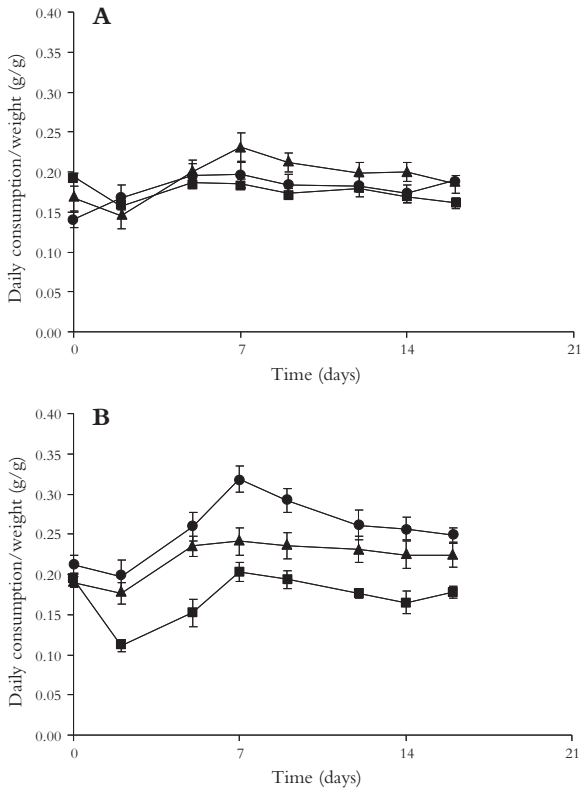

Figure 2. **Average daily consumption in grams chow per bodyweight of mice receiving control (squares), phytol enriched (triangles) or clofibrate enriched diets (circles)**. Panel A: *Pc-tp* (-/-) mice. Panel B: *Pc-tp* (+/+) mice. Data are the mean of five mice (± S.E.M).

the food consumption was normal. The *Pc-tp* (-/-) mice did not show any morphological signs of obesity. Diets containing 0.5% (w/w) clofibrate or phytol were administered to the *Pc-tp* (-/-) and *Pc-tp* (+/+) mice for a period of three weeks. The relative body weight development of the (-/-) and (+/+) mice was similar on each diet and tended to increase (figure 1), probably to make up for the initial body weight loss (see experimental procedures). The food consumption however, was not similar. *Pc-tp* (+/+) mice on clofibrate and phytol supplemented diets had a significantly higher average daily food intake as compared to the food intake of (+/+) mice on the control diet and (-/-) mice on all three diets (figure 2). Thus, despite a higher food consumption, *Pc-tp* (+/+) mice do not gain weight, probably due to clofibrate and phytol-induced enhanced lipid metabolism.

Pc-tp expression in the livers of clofibrate and phytol-fed mice

Since the promoter sequence of *Pc-tp* contains a PPAR/RXR binding site, we investigated the PC-TP expression in liver under the influence of the PPARα activators clofibrate and phytol. As shown in figure 3 (lower panel, lanes 2 and 3) these activators increased PC-TP levels in the liver of *Pc-tp* (+/+) mice. By densitometric scanning of these immunoblots (n=4) and by comparing the densities with a PC-TP calibration curve, it was estimated that the amount of PC-TP increased from 0.21 ± 0.13 ng to 0.74 ± 0.22 ng (3.5-fold) and 1.48 ± 0.20 ng (7.0-fold) PC-TP per mg of total liver cytosol protein in the phytol and clofibrate groups, respectively. Under these feeding conditions, PC-TP could not be detected in *Pc-tp* (-/-) mice (data not shown). To ascertain that the PPARα activators did act in *Pc-tp* null mice the inducible peroxisomal acyl-CoA oxidase was visualized by immunoblotting. As shown in figure 3 (upper and middle panel) the phytol and clofibrate diets increased the acyl-CoA oxidase levels in both the *Pc-tp* (-/-) and (+/+) mice to comparable extents

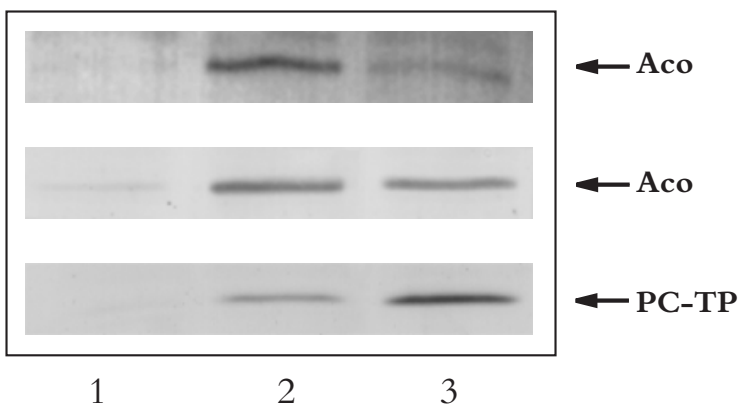

Figure 3. **Immunoblot analysis of PC-TP and acyl-CoA oxidase (Aco) in the livers from *Pc-tp* (-/-) and *Pc-tp* (+/+) mice fed regular chow (lane 1), phytol- (lane 2) or clofibrate- (lane 3) enriched chow for three weeks.** Upper panel: Aco levels in *Pc-tp* (-/-) mice. Middle Panel: Aco levels in *Pc-tp* (+/+) mice. Lower panel: PC-TP levels in *Pc-tp* (+/+) mice.

Lipids in blood plasma

Changes in body weight are often accompanied by changes in plasma lipid profile (26). Here we determined the total triglyceride (TG), cholesterol and phospholipid levels in blood plasma of *Pc-tp* (-/-) and (+/+) mice fed with the clofibrate- and phytol-supplemented diets for a period of three weeks. In the *Pc-tp* null mice, the TG

levels were slightly increased as compared to *Pc-tp* (+/+) mice (figure 4a). Clofibrate administration gave rise to a significant decrease in the TG levels of *Pc-tp* (+/+) mice; this was not observed for *Pc-tp* (-/-) mice. The effect of phytol administration was less pronounced. The difference in TG levels between the (-/-) and (+/+) mice was 32% for the clofibrate-, 20% for the phytol- and 15% for the control diet. Cholesterol levels appeared to be affected by these diets as well, but this was the case in both the *Pc-tp* (-/-) and *Pc-tp* (+/+) mice (figure 4b). As for the phospholipid levels, we found that clofibrate administration gave rise to a significant reduction in the *Pc-tp* (+/+) mice, which was not observed in the *Pc-tp* null mice (figure 4c). The effect of the phytol diet on the phospholipid levels was less pronounced. The mean difference in phospholipid levels between *Pc-tp* (-/-) and (+/+) mice was 32% for the clofibrate, 16% for the phytol and 15% for the control diet. Analysis of the blood plasma phospholipid classes showed that the observed decreases were the same for all phospholipid classes (data not shown).

In agreement with a previous study (27) we found that clofibrate feeding increased the PC levels by 20 % and decreased the TG levels by 23% in the livers of *Pc-tp* (+/+) mice. Similar effects on liver lipid levels were observed for the *Pc-tp* null mice (data not shown). Histological analysis of liver, kidney and intestine revealed no aberrations in the PC-TP *Pc-tp* (-/-) and (+/+) mice.

Table 1. **PC molecular species in blood plasma of *Pc-tp* (-/-) and *Pc-tp* (+/+) mice fed control and phytol or clofibrate supplemented diets for three weeks.** Data represent means of 3 to 5 mice (± S.E.M.).

sn-1, *sn*-2	Control		Phytol		Clofibrate	
	Pc-tp (-/-)	Wild type	*Pc-tp* (-/-)	Wild type	*Pc-tp* (-/-)	Wild type
			(mol% of total species)			
16:0, 16:0	1.0 ± 0.1	0.8 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.3 ± 0.3
16:0, 18:1	12.8 ± 0.4	11.5 ± 0.3	23.5 ± 2.4	27.2 ± 2.4	17.6 ± 1.2	17.9 ± 3.5
16:0, 18:2	36.4 ± 1.5	33.3 ± 2.0	23.4 ± 2.0	24.4 ± 0.1	34.7 ± 0.9	29.6 ± 4.6
16:0, 20:3/18:0, 18:3	6.4 ± 0.2	5.5 ± 0.5	9.7 ± 0.3	7.2 ± 0.6	12.0 ± 0.3	8.8 ± 0.1
16:0, 20:4	7.8 ± 0.2	7.5 ± 1.0	7.2 ± 1.0	6.5 ± 0.6	5.9 ± 0.7	7.9 ± 1.1
16:0, 22:6	7.2 ± 0.5	9.4 ± 1.2	7.3 ± 0.8	6.7 ± 0.3	6.9 ± 0.6	5.9 ± 0.1
18:0, 18:1	3.6 ± 0.2	3.7 ± 0.0	4.5 ± 0.3	5.0 ± 0.2	2.5 ± 0.1	4.0 ± 0.1
18:0, 18:2/18:1, 18:1	15.6 ± 0.4	17.4 ± 1.1	11.5 ± 0.6	12.3 ± 0.3	10.8 ± 0.4	16.3 ± 1.1
18:0, 20:3	.3 ± 0.1	2.3 ± 0.2	3.7 ± 0.4	3.2 ± 0.4	3.2 ± 0.3	2.5 ± 0.1
18:0, 20:4	4.6 ± 0.3	5.2 ± 0.9	5.5 ± 1.0	4.2 ± 0.4	3.5 ± 0.5	4.6 ± 0.8
18:0, 22:6	2.3 ± 0.3	3.3 ± 0.4	2.4 ± 0.2	2.1 ± 0.2	1.8 ± 0.1	1.2 ± 0.0

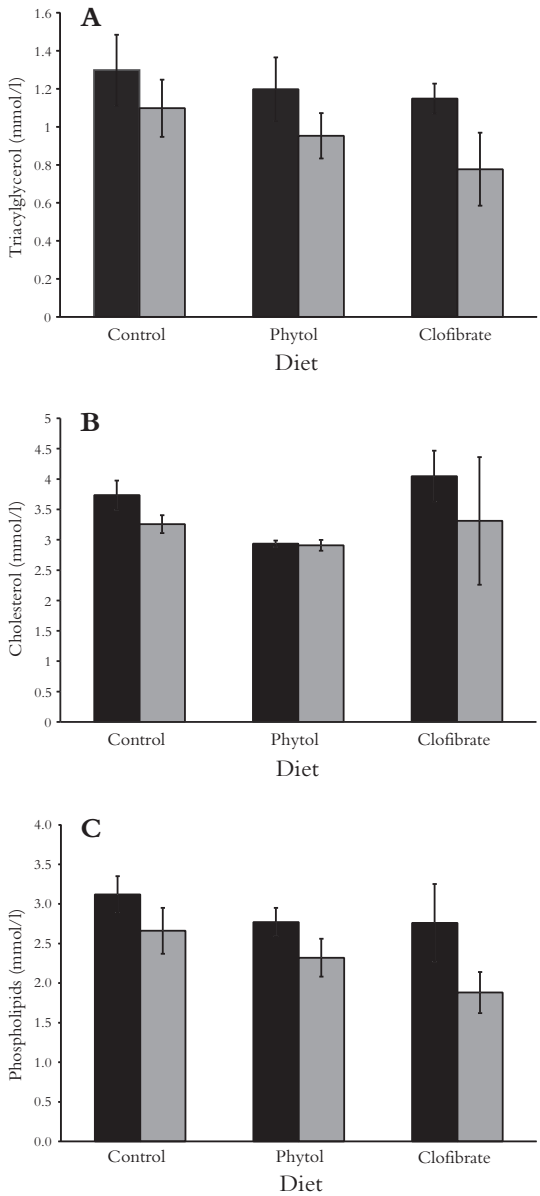

Figure 4. **Triglyceride, cholesterol and phospholipid levels in blood plasma of *Pc-tp* (-/-) and *Pc-tp* (+/+) mice fed phytol- and clofibrate-enriched chow for three weeks.** Panel A: TG; panel B: cholesterol; panel C: phospholipid. *Pc-tp* (-/-) mice are represented by black bars and *Pc-tp* (+/+) mice by grey bars. Data represent means of 3 to 5 mice (± S.E.M.).

PC molecular species in blood plasma

The PC species composition in blood plasma of *Pc-tp* (+/+) and *Pc-tp* (-/-) mice was not significantly different with C16:0/C18:2- and C18:0/C18:2-PC as the major species (table 1, control, phytol). This indicates that PC-TP has no effect on the PC species composition. Even after a significant decrease of total PC levels in the plasma of *Pc-tp* (+/+) mice as a result of clofibrate treatment (figure 4) no significant change was observed in the PC species profile of the two groups of mice (table 1, clofibrate). Similarly, no significant changes were observed in the molecular species composition of PE and SM (data not shown).

Discussion

So far, despite extensive efforts the physiological function of PC-TP has been very elusive. Recently, the structure of rat genomic DNA was elucidated (16). The promotor region contained a number of responsive elements among which that for the PPAR/RXR heterodimer. The availability of the *Pc-tp* null mice offered the opportunity to establish whether there was a relationship between PPAR activators and the expression of PC-TP. Our data show that a diet containing the PPARα activators clofibrate and phytol increased the levels of liver PC-TP of *Pc-tp* (+/+) mice by 3.5 and 7.5-fold, respectively.

PPARα activators are known for their hypolipidemic effect. It is thought that both enhanced plasma triglyceride clearance and decreased hepatic secretion of very-low-density-lipoprotein (VLDL) contribute to this process (28). In agreement with these observations, we have found that the clofibrate and phytol diets significantly reduced TG and phospholipid levels in blood plasma of *Pc-tp* (+/+) mice but not of *Pc-tp* (-/-) mice. Thus, it appears that there is a connection between the upregulation of PC-TP and the reduced levels of these lipids in blood plasma. The increased body weight of *Pc-tp* (-/-) mice by 15% as compared to the *Pc-tp* (+/+) mice supports this hypothesis, since an increase in body weight is closely related to higher plasma triglyceride and phospholipid levels (26, 29). In addition, the *Pc-tp* (-/-) mice on the clofibrate diet maintained this overweight, despite a significantly lower daily food intake than the *Pc-tp* (+/+) mice. Interestingly, *PPARα* null mice have increased body weights as well (29). These mice gain 22% more weight during their first seven months of growth. In this regard it is to be noted that the PPARα-activators act normally in the *Pc-tp* (-/-) mice as shown by the upregulation of acyl-coA oxidase in the liver. In contrast to *PPARα* null mice, *Pc-tp* null mice did not show any clear

morphological signs of obesity. It seems that the absence of PC-TP attenuates lipid homeostasis only subtly.

At this point we do not know how PC-TP affects lipoprotein metabolism. However, we have observed that PC-TP is rapidly relocated to mitochondria of endothelial cells under the influence of clofibrate in the medium (chapter 4). Hence, it could be possible that PC-TP plays a regulatory role in mitochondrial fatty acid oxidation, resulting in reduced plasma TG and phospholipid levels. On the other hand, these results also support a role for PC-TP as regulator of the phosphatidylethanolamine methyltransferase (PEMT)(chapter 4). Inhibition of PEMT impairs the lipidation of apolipoprotein B48-containing lipoproteins, leading to decreased TG and phospholipid levels in blood (30). However, the PE methylation pathway appears to be restricted to liver (31), whereas the relocation of PC-TP has only been established in endothelial cells.

Previously, it was proposed that PC-TP plays a role in the bile formation by transporting PC to the site of secretion (5, 8, 9). In support of this, the PC species for which PC-TP expressed a preference were found in bile (10). By perfusion of isolated rat livers with or without the bile salt taurocholate, it was inferred that the PC species in bile and high-density lipoprotein (HDL) originate from the same hepatic pool or were made available by the same mechanism (32). Here we show that the phospholipid and PC species composition of plasma lipoproteins from wild type and *Pc-tp* (-/-) mice is very similar. This, together with *Pc-tp* null mice having normal bile secretion (chapter 6), implies that PC-TP is not involved in the secretion of PC by the liver or other tissues.

In this study we have obtained the first evidence for a phenotype of the *Pc-tp* null mice. It appears that these mice are less sensitive to PPARα activators known to have profound effects on lipid homeostasis. Though the exact mechanism remains to be established, our data show that PC-TP is involved in the PPARα activator-induced hypolipidemia, indicating that PC-TP belongs to the family of PPARα–controlled proteins involved in intracellular transport and degradation of fatty acids.

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Chapter 8

Summarizing discussion

PC-TP has been studied for over 30 years now. Despite extensive research concerning the biochemical, biophysical and structural properties of PC-TP, the function of this protein is still elusive. The mechanism by which PC-TP extracts a PC molecule from a membrane is largely unknown. Furthermore, convincing evidence in support of a physiological function is still lacking. We have studied *in vitro* the folding and the mechanism of PC extraction from membranes. *In vivo*, we have assessed previously postulated and novel physiological functions of PC-TP by localization studies in various cell lines and by examining lipid metabolism in cells overexpressing PC-TP and *Pc-tp* null mice.

Expression in *Escherichia coli*

In a previous study it was reported that bovine PC-TP could not be expressed in *Escherichia coli* (1). It was inferred that the expression of PC-TP required PC, and that the absence of PC in these bacteria was the limiting factor. However, the same authors successfully expressed human PC-TP in this prokaryote (2). This expression was 3-fold enhanced by redesigning the open reading frame according to preferred codon usage for *E. coli*. We were able to express bovine PC-TP in *E. coli*. Expression levels were similar to those of the redesigned human PC-TP. This indicates that bovine PC-TP does not need remodelling to reach adequate expression levels. In case of the murine PC-TP this would still be useful, since the expression was 8-fold lower. The rapid isolation procedure of PC-TP expressed in *E. coli* and the accessibility of this organism to genetic engineering, provides us with a useful tool to investigate the mechanism of PC exchange by site-directed mutagenesis or the structure of PC-TP by X-ray crystallography.

Folding of PC-TP

The expression of recombinant proteins in *E. coli* can result in the formation of inclusion bodies in the cell cytoplasm or periplasm (3, 4). These inclusion bodies are insoluble aggregates of the recombinant protein resulting from the accumulation of folding intermediates. Approximately 90% of the recombinant PC-TP accumulates in inclusion bodies. This PC-TP can be activated using standard refolding techniques (for reviews: (5, 6)) with PC as additional component. Since recombinant PC-TP purified from cytosol contains a PE or PG in a one-to-one complex, it seems that other phospholipids can substitute for PC in the absence of the latter. Recently, phospholipids were also shown to be essential in the folding of phosphatidylinositol transfer protein isoforms from inclusion bodies (7). Both *in vitro* and *in vivo* phospholipids may be required to shield hydrophobic segments of phospholipid

transfer proteins from the aqueous environment so as to prevent aggregation and to allow the protein to adopt the proper tertiary structure.

Mechanism of transfer

PC-TP is representative for the START domain found in a number of lipid transporting proteins (8, 9). In analogy to the crystal structure of the START domain of human MLN64, PC-TP can be folded as such to form a hydrophobic tunnel accommodating PC. Accordingly, the polar head group is proposed to interact with the Arg^{78} and Asp^{82} present in a loop at the surface of the protein (9). Previous studies showed that the two acyl chains of PC are positioned separately inside the PC-TP molecule (10). Tyr^{54} and Val^{171}-Asn^{177} are part of the peptide segments that accommodate the *sn*-2 acyl chain (11-13). In line with this, modification of the Lys^{55} positioned right next to the Tyr^{54} by the apolar reagent phenylisothiocyanate inhibited PC transfer activity (14). We confirmed by site-directed mutagenesis that this amino acid residue is indeed essential for PC transfer activity. Since this amino acid residue is buried deep inside the binding pocket, Lys^{55} is likely to play a role during the exchange of PC at a membrane interface. This is supported by the fact that the inhibition by chemical modification is enhanced in the presence of vesicles.

We propose the following model for PC-TP-mediated PC exchange at a membrane interface. Upon arrival at a membrane the α-helical loop at the N-terminus of PC-TP interacts with the membrane interface (2). Subsequently, the hydrophobic region between Trp^{186}-Ala^{192} can penetrate the membrane bilayer (2, 15). This leads to exposure of the hydrophobic lipid-binding site to the membrane interface. The lysine residue present in the lipid-binding site is needed to neutralise the negatively charged phosphate groups of the phospholipids in the membrane facilitating the exchange of PC. After exchange, PC-TP retreats from the interface and closes again.

PC species preference

PC-TP is found to bind preferentially C16:0/PUFA-PC when in contact with a membrane. Proteins and other phospholipids present in membranes of the endoplasmic reticulum and Golgi apparatus or in the plasma membrane do not seem to affect this preference. This preference can be caused by the intrinsic properties of the lipid-binding site of PC-TP or reflect the ease with which C16:0/PUFA-PC can be released from the membrane. Membranes containing high levels of unsaturated phospholipids are less tightly packed (for review: (16)). A shorter acyl chain on the *sn*-1 position contributes to this loss in membrane coherence. Consequently,

C16:0/PUFA-PC species can be extracted more easily from the membrane than the C18:0/PUFA-PC species. This agrees with the observation that an increase in membrane fluidity enhances PC-TP-mediated PC transfer (17, 18). Using ESI-TOF, we have found that PC-TP containing C16:0/PUFA-PC is less stable than C18:0/PUFA-PC. Taken together, these results indicate that the uptake of PC species by PC-TP is regulated by the ease with which molecular species of PC leave the membrane. The release is determined by the interaction between PC-TP and PC. Higher uptake and release rates of C16:0/PUFA-PC eventually lead to accelerated exchange of especially this species.

Relocation of PC-TP by phosphorylation

PC-TP is evenly distributed throughout the cytoplasm of FBHE cells, HUVEC, HepG2 cells and NIH3T3 mouse fibroblasts. As measured by fluorescence recovery after bleaching, PC-TP was found to be highly mobile throughout the cell. After the addition of clofibrate, PC-TP relocated to mitochondria in endothelial cells within 5 min. This process is probably mediated by phosphorylation of Ser^{110} present in the sole putative PKC phosphorylation site of PC-TP. Fibrates are reported to induce phosphorylation of serine and threonine residues (19, 20) by protein kinase C (PKC) (21-24, 25, 26). If PKC is involved in the phosphorylation of PC-TP, it is probably a specific isoform, since phorbol ester that directly stimulates PKCα,β_1,γ and δ (27, 28) did not induce relocation. This is supported by the observation that rat brain PKC consisting mainly of PKCγ was not able to phosphorylate PC-TP. The effects of clofibrate and PMA on phospholipid metabolism are indeed reported to be mediated by separate PKC isoforms (29).

PC-TP and PPARα

Clofibrate is a well-known PPARα activator (30, 31). The PPARα nuclear receptor is regarded as a general regulator of lipid metabolism-related enzymes controlling lipid homeostasis (for reviews: (32-35)). The major intracellular target of PPARα is the β-oxidation in peroxisomes and mitochondria (36) whereas extracellularly it affects lipoprotein metabolism. These two processes do not occur independently of each other, since changes in β-oxidation affect the lipidation of lipoprotein particles. PC-TP is under the control of PPARα as well. The promoter of PC-TP contains a PPARα responsive element and consequently PC-TP levels in mouse liver were 7.5-fold elevated after clofibrate feeding. In addition, the presence of PC-TP was indispensable for mice to respond properly to clofibrate administration. In combination with the clofibrate-induced relocation to

mitochondria, it seems logical to focus our attention to PPARα activator-mediated effects on mitochondria. This is supported by the observation that PPARα deficiency leads to an increase in body weight of 22% (37) as compared to 15% for the *Pc-tp* null mice. This indicates that PC-TP is a moderator of a part of the reported deviations caused by PPARα deficiency.

PC-TP as inhibitor of PEMT

Clofibrate it is an inhibitor of PEMT that mediates the methylation of PE to PC, a process that takes place in the mitochondria-associated membranes (38, 39). Inhibition of PEMT impairs the lipidation of apolipoprotein B48-containing lipoproteins, leading to decreased TG and phospholipid levels in blood (40). In contrast, clofibrate treatment did not affect blood plasma TG and phospholipid levels in *Pc-tp* null mice. In view of its association with mitochondria after clofibrate stimulation, PC-TP could be a regulator of PEMT. On the other hand, the PE methylation pathway appears to be restricted to liver (41), whereas PC-TP is more ubiquitously expressed.

PC-TP and mitochondrial β-oxidation

PC-TP can be defined as a non-membranous pool of C16:0/PUFA-PC species, since preferably these PC species are transported. It is conceivable that relocation of PC-TP to mitochondria causes an increase of these species in (parts of) the mitochondrial membranes. Mitochondrial function can be modulated by altered lipid profiles. An increase in membrane fluidity results in a decrease of the succinate-quinone oxidoreductase (SQR) (42) and pyruvate carrier activity (43, 44). SQR catalyses the oxidation of succinate to fumarate in the citric acid cycle and donates the electrons to quinone in the membrane, thereby connecting the citric acid cycle to the respiratory chain (for review: (45)). The pyruvate carrier transports pyruvate across the mitochondrial membrane into the matrix (for review: (46)). In addition, changes in mitochondrial membrane fatty acid composition and cause proton leakage (47, 48) and Ca^{2+} efflux (49), affecting the proton motive force and membrane potential in mitochondria. Taken together, this infers that an increase in membrane fluidity causes a decrease in energy derived from glycolysis. To compensate for this energy drain, usually more fatty acids are degraded. So, by releasing the C16:0/PUFA-PC species in the mitochondrial membranes, PC-TP increases the fluidity of this membrane leading to a stimulated β-oxidation. Since a stimulated β-oxidation is proposed to be one of the main causes of decreased lipoprotein secretion, this can account for the relatively high levels of TG and

phospholipids in the blood plasma of PC-TP null mice.

PC-TP and mitochondrial biogenesis

PPARα activators induce the biogenesis of mitochondria, albeit to a much lesser extent as the proliferation of peroxisomes (50, 51). These newly formed mitochondria need specific proteins and lipids originating from outside the mitochondria to function properly. The outer and inner membrane contact sites play a role in the import of these mitochondrial proteins and lipids (52, 53). Unsaturated fatty acyl chains are required in outer membrane contact sites of mitochondria (54). By changing or maintaining the lipid profile of these contact sites, PC-TP could play a role during the biogenesis of mitochondria. An increase in mitochondria leads to increased β-oxidation, which in turn decreases TG and phospholipid levels in blood plasma. In addition, the mitochondrial contact sites regulate mitochondrial metabolism (55, 56) and the channelling metabolites and ions into the inner compartment (56, 57) thereby also being involved in the regulation of the energy status of mitochondria.

PC-TP and the assembly of lipoprotein particles

Pc-tp null mice had a higher level of TG and phospholipids in their blood plasma, which infers a change in lipoprotein content. Three major lipoproteins have been described in blood plasma: the chylomicrons, VLDL/IDL/LDL and HDL (for review: (58)). The liver plays an essential role in the biogenesis of VLDL and HDL, whereas the chylomicrons are formed in the intestine. Since PC-TP is expressed highly in liver and intestine, PC-TP could be involved in the assembly of lipoprotein particles. *Pc-tp* (+/+) mice had a higher level of TG and phospholipids in their blood plasma. The lipoproteins chylomicrons/VLDL and HDL are known to transport mainly TG and cholesterol, respectively (for reviews: (58-60)). Since the absence of PC-TP seems to affect the TG levels rather than the cholesterol levels, this could indicate that mainly the chylomicrons and VLDL particles are affected by PC-TP deficiency. This could be by means of the earlier mentioned PEMT, but also in PEMT independent ways. Reduced secretion of VLDL-triglycerides can be caused by inhibition of TG synthesis, which in turn can occur by modulation of the membrane lipid species composition (61). Furthermore, there is evidence for the involvement of membrane lipid species composition on the degradation of apo-B (62). PC-TP might be involved in changing the membrane species composition thereby inhibiting TG synthesis or stimulating the degradation of apo-B, leading to a decreased VLDL content.

Final conclusion

It is clear that the physiological function of PC-TP is related to the effects of PPARα activators on lipid metabolism in the body. Since PC-TP relocates to mitochondria, it is inevitable to focus attention to this organelle. We think that the main target of PC-TP is the β-oxidation of fatty acids. At an influx of an excess of fatty acids, PC-TP relocates to mitochondria and stimulates the β-oxidation. At the same time, PC-TP is upregulated via its PPARα responsive element. The next time an excess of fatty acids is presented to the cell, the cell can cope more effectively with these lipids. In the long run this leads to the reduced TG and phospholipid levels in blood plasma. Besides modulating the β-oxidation, PC-TP could also inhibit PEMT. Other possibilities include the inhibition of TG synthesis and stimulation of apo-B lipoprotein degradation. The main hypotheses are summarised in the following figure:

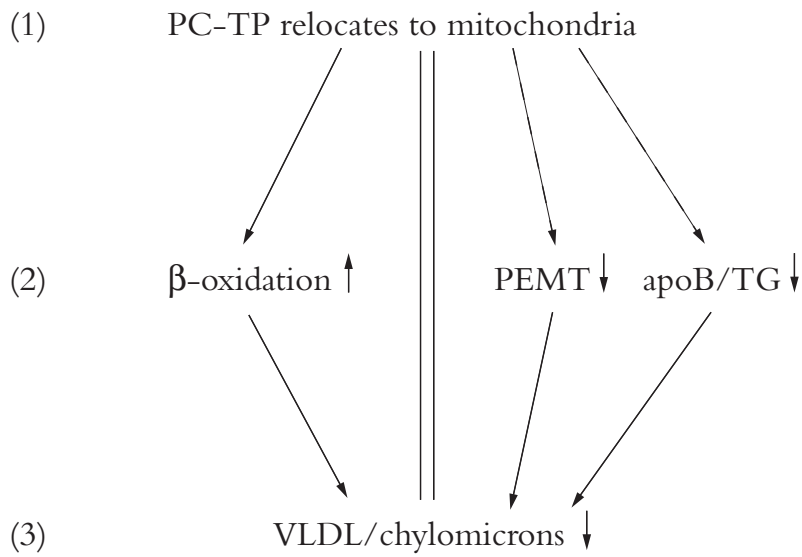

Figure 1. **Possible mechanisms of action of PC-TP in the cell.**

To test these possibilities, a lot of research still has to be done. Since PC-TP null mice are available (to everyone !), as well as the proper biomolecular tools, it seems only to be a matter of time before the physiological function of PC-TP is unveiled; and this more than 30 years after its discovery.

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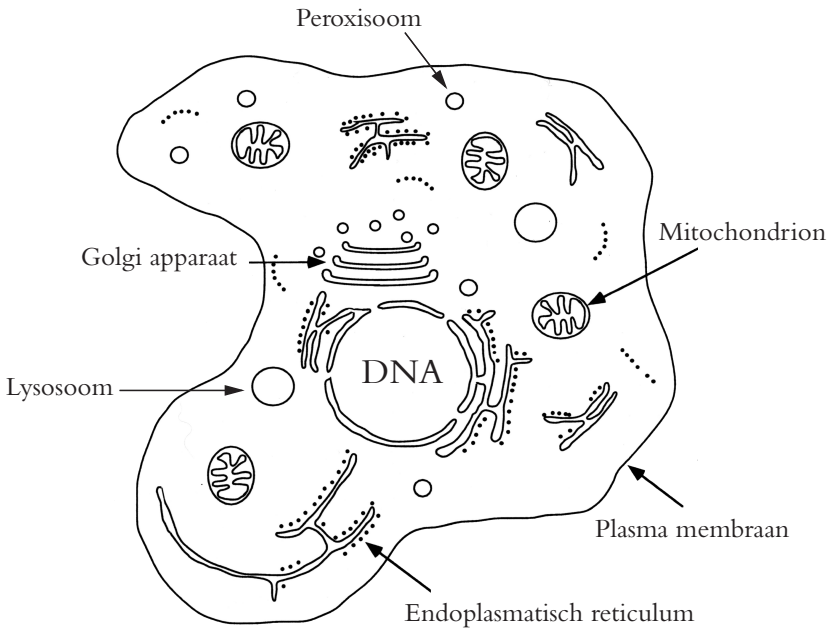
Samenvatting in het Nederlands



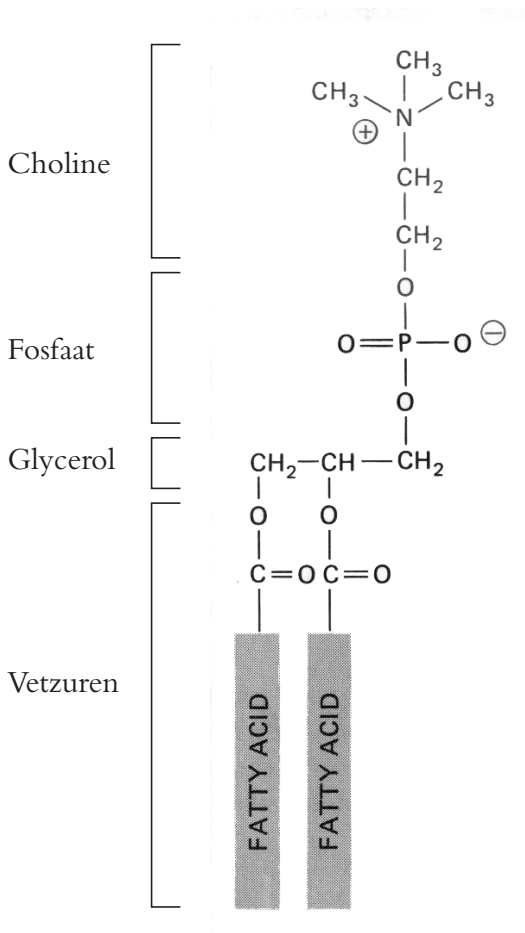
Figuur 1. **Een dierlijke cel.**

Het menselijk lichaam is opgebouwd uit cellen. Een cel bestaat uit verschillende compartimenten, zoals de kern, het cytoplasma, het endoplasmatisch riticulum, het Golgi apparaat, mitochondriën, lysosomen en peroxisomen (figuur 1). Elk van deze delen van de cel hebben een specifieke functie. In de kern bevindt zich het erfelijk materiaal, het DNA. Dit DNA wordt in de kern vertaald in boodschappers (het RNA), die uiteindelijk zorgen dat er eiwitten worden gemaakt in het cytoplasma of aan het endoplasmatisch reticulum. Deze eiwitten kunnen dan in het cytoplasma het werk doen of via het Golgi apparaat de cel uit gezonden worden. De mitochondriën zijn de energiefabriekjes van de cel. In lysosomen en peroxisomen wordt alles wat niet meer nodig is in de cel afgebroken.

De inhoud van de compartimenten bestaat voor het grootste deel uit water. Vandaar dat meer dan 80% van je lichaam uit water bestaat. De waterige inhoud van een compartiment wordt omgeven door een laagje vet, het membraan. Membranen vormen de grens tussen de verschillende compartimenten. Ze bestaan uit verschillende vetmoleculen waaronder de fosfolipiden (vet met een fosfaat groep) en

het meer bekende cholesterol. Deze vetten lunnen van het ene naar het andere membraan vervoerd worden door middel van contact tussen membranen, maar ook via water oplosbare eiwitten. Het phosphatidylcholine transfer protein (PC-TP) is een water oplosbaar eiwit dat in staat is om specifiek phosphatidylcholine (PC), één van de fosfolipiden, te verplaatsen van de ene naar de andere membraan. Tenminste 30% van alle vetten in de membranen in de cel bestaat uit PC. Vooral bij celdeling zijn grote hoeveelheden PC nodig om als bouwstenen voor nieuwe membranen te dienen. Eerst werd gedacht dat PC-TP hier een kleine bijdrage aan leverde, maar dat bleek niet het geval te zijn (hoofdstuk 5 en 6).

PC bestaat uit een glycerol skelet met daaraan een fosfaat met een choline hoofdgroep en twee lange vetzuurstaarten (figuur 2). Er kunnen verschillende



Figuur 2. **Een fosfatidylcholine molecuul.**

vetzuurstaarten aan een PC molecuul vast zitten, zoals de onverzadigde vetzuren linolzuur en arachidonzuur of het verzadigde palmitinezuur. PC-TP transporteert niet alle PC moleculen even snel, PC met een palmitinezuur (C16:0) en arachidonzuur (C20:4) wordt het snelst vervoerd (hoofdstuk 3). Dat komt waarschijnlijk doordat deze PC moleculen veel losser in de membraan zitten dan andere PC moleculen waardoor PC-TP deze gemakkelijker kan pakken. Omdat deze PC moleculen vervolgens slechter aan PC-TP vastgeplakt zitten, worden ze ook weer snel afgegeven aan de volgende membraan die PC-TP tegen komt.

Zoals boven al beschreven heeft elk compartiment in de cel zijn eigen functie. Bij die functie horen specifieke eiwitten, maar ook specifieke vetten. Nu is het wel zo dat alle membranen in de cel voor de helft uit PC bestaan, maar sommige membranen of zelfs gebiedjes in deze membranen bestaan uit specifieke PC moleculen, zoals C16:0/C20:4-PC. Zou PC-TP deze PC moleculen naar een specifieke plek in de cel vervoeren ? Om PC-TP te kunnen volgen op zijn weg door de cel, hebben we het PC-TP zichtbaar gemaakt door er een fluorescente groep aan te hangen (hoofdstuk 4). Het eiwit krijgt daardoor een kleurtje zodat je met behulp van geavanceerde technieken kunt zien waar het eiwit in de cel is. Het is zelfs mogelijk om dit te doen in levende cellen buiten het lichaam. Het bleek dat PC-TP in de hele cel te zien is en zich heel snel kan verplaatsen door de cel. Om nu PC-TP naar één van de compartimenten te verplaatsen werden de cellen gepest met behulp van een aantal chemische stoffen om zo de verdeling van PC-TP in de cel te beinvloeden. Eén van de geteste stofjes, clofibraat, zorgde ervoor dat PC-TP binnen vijf minuten naar de mitochondriën ging.

Clofibraat verlaagt de vetten in het bloed door in de lever de afbraak van vetten te stimuleren. De lever is het belangrijkste orgaan in ons lichaam dat de hoeveelheid vet in het bloed regelt. Mitochondriën (de energiefabriekjes) spelen een belangrijke rol bij deze vetverbranding. PC-TP komt veel voor in de lever en gaat naar de mitochondriën. Kan PC-TP misschien iets te maken hebben met deze vetverbranding ? Dankzij het Nederlands Kanker Instituut hadden we een goed model om dit idee te testen. Er waren namelijk muizen gemaakt zonder PC-TP. Deze muizen werden clofibraat gevoerd en vervolgens vergeleken met normale muizen (hoofdstuk 7). De muizen zonder PC-TP konden het gehalte aan vet in het bloed niet verlagen en normale muizen wel. Na toediening van clofibraat stimuleert PC-TP dus de afbraak van vetten, waarschijnlijk door C16:0/C20:4-PC af te geven aan de mitochondriën. Ondanks dat we niet precies weten hoe, heeft PC-TP iets te maken heeft met de afbraak van vetten in lever en misschien ook de rest van het lichaam.

Dankwoord

Ondanks de kaft was ik toch van plan deze en gene te eren in een dankwoord. Allereerst wilde ik Karel bedanken voor de mogelijkheid die hij mij gegeven heeft om dit onderzoek succesvol af te ronden. Ik weet dat je lange tijd getwijfeld hebt over het voortzetten van dit project, maar ik denk dat we er zo toch nog een mooi geheel van gemaakt hebben. Verder wilde ik zeker niet mijn copromotor vergeten. Ben, omdat het onderzoek een heel andere richting uitging dan we voorspeld hadden, heb je misschien minder praktische inbreng gehad dan je zelf gehoopt had. Je bleek het echter feilloos aan te voelen als ik het weer wat moeilijk had. Jouw wijze raad (en anekdotes) hebben het allemaal een stuk lichter gemaakt.

Dan is het nu tijd om een woordje van dank aan mijn studenten te richten. Susanne, jij was mijn eerste echte stagiaire. Daarnaast was je ook mijn uitlaatklep. Het is dat je me goed kon inschatten, want andere mensen hadden aan mijn opmerkingen misschien wel een klein trauma overgehouden. We gaan elkaar nog vaak zien. Mijn andere paranimf Loesje, is de tweede die een uitzonderlijke bijdrage heeft geleverd aan dit boekje. Je komt misschien nogal wat naïef en onzeker over, maar je mag jezelf nooit meer onderschatten. Wat Susanne heeft betekend voor mijn geestelijke gesteldheid, heb jij gedaan voor de wetenschappelijke inhoud van dit boekje. Verder wilde ik Anne ook nog roemen. Je bent misschien niet de meest extroverte persoon die ik ken, maar wel een van de slimste. Je gaat het vast en zeker maken bij massa spectrometrie. Als laatste in het rijtje hoort Sebastiaan. Ik heb je wel niet volledig begeleid, maar het onsterfelijke voorval met je haar, zal ik nooit vergeten en vaak navertellen (gelukkig dat het geen vervelende gevolgen voor je had).

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Als laatste. Als allerlaatste. Als allerliefste. Onvoorwaardelijk. Deze zoen is voor jou, Conny. Beter een idealist dan een cynicus. Liever vergeven en dan vergeten. Het was soms moeilijk, meisje, maar dat is nu voorbij. En nu ga ik stoeien met Merijn.

List of Publications

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Curriculum Vitae

Arjan de Brouwer werd op 13 april 1972 geboren te Heesch. In soepele tred werden peuter-, kleuter-, and basischool doorlopen. In 1989 werd het gymnasium diploma behaald aan het Titus Brandsma Lyceum te Oss. Hierna werd de studie biologie opgepakt aan de Katholieke Universiteit Nijmegen en afgerond in augustus 1996 in de toegepaste en de biochemisch-fysiologische afstudeerrichtingen. Vanaf oktober 1996 werd enthousiast gewerkt aan een promotie binnen de vakgroep Biochemie van Lipiden aan de Universiteit Utrecht. In dit boekje staan de wederkerigheden gedurende deze laatste periode opgetekend. Momenteel is hij als postdoc verbonden aan de afdeling Antropogenetica van het Universitair Medisch Centrum St. Radboud met als onderwerp de genetische achtergronden van doofheid.