REVIEW ARTICLE

Phospholipid transfer proteins revisited

Karel W. A. WIRTZ

Institute of Biomembranes, Centre for Biomembranes and Lipid Enzymology, Utrecht University, P.O. Box 80054, 3508 TB Utrecht, The Netherlands

Phosphatidylinositol transfer protein (PI-TP) and the non-specific lipid transfer protein (nsL-TP) (identical with sterol carrier protein 2) belong to the large and diverse family of intracellular lipid-binding proteins. Although these two proteins may express a comparable phospholipid transfer activity *in vitro*, recent studies in yeast and mammalian cells have indicated that they serve completely different functions. PI-TP (identical with yeast SEC14p) plays an important role in vesicle flow both in the budding reaction from the *trans*-Golgi network and in the fusion reaction with the plasma membrane. In yeast, vesicle budding is linked to PI-TP regulating Golgi phosphatidylcholine (PC) biosynthesis with the apparent purpose of maintaining an optimal PI/PC ratio of the Golgi complex. In mammalian cells, vesicle

flow appears to be dependent on PI-TP stimulating phosphatidylinositol 4,5-bisphosphate (PIP₂) synthesis. This latter process may also be linked to the ability of PI-TP to reconstitute the receptor-controlled PIP₂-specific phospholipase C activity. The nsL-TP is a peroxisomal protein which, by its ability to bind fatty acyl-CoAs, is most likely involved in the β -oxidation of fatty acids in this organelle. This protein constitutes the N-terminus of the 58 kDa protein which is one of the peroxisomal 3-oxo-acyl-CoA thiolases. Further studies on these and other known phospholipid transfer proteins are bound to reveal new insights in their important role as mediators between lipid metabolism and cell functions.

INTRODUCTION

Phospholipid transfer proteins (PL-TPs) are known for their ability to act *in vitro* as specific carriers of single phospholipid molecules between membranes. These proteins have been detected in all eukaryotic cells investigated to date. The best known PL-TPs purified from mammalian tissues are phosphatidylcholine transfer protein (PC-TP), which is highly specific for phosphatidylcholine (PC) [1], PI-TP, which expresses a distinct preference for phosphatidylinositol (PI) [2], and the non-specific lipid transfer protein (nsL-TP), which transfers all common phospholipids, but also cholesterol and glycolipids [3,4]. Although many studies were carried out on the properties of these proteins and their expression in cells under various physiological conditions [5–7], their actual cellular function remained very much an enigma. During the last few years, however, some very interesting observations have clearly indicated that PL-TPs are involved in some fundamental processes. So it was realized that SEC14p, a protein required for the export of yeast secretory proteins from the Golgi complex, had all the features of a PI-TP [8,9]. In line with this, components identified as being essential both for the ATP-dependent priming of Ca²⁺-activated secretion and for the budding of vesicles from the *trans*-Golgi network were shown to be identical with PI-TP [10,11]. In addition, G-protein-stimulated phospholipase C (PLC) activity was found to be critically dependent on PI-TP as well [12]. From previous studies it was inferred that nsL-TP (also called ‘sterol carrier protein 2’, SCP₂) was involved in various aspects of intracellular cholesterol metabolism [13,14]. Now we know that nsL-TP, together with a

related 58 kDa protein, is prominently present in peroxisomes [15] and that these proteins play a key role in the peroxisomal β -oxidation of fatty acids ([16]; F. S. Wouters, P. I. H. Bastiaens, K. W. A. Wirtz and T. M. Jovin, unpublished work). This review will focus on PI-TP and nsL-TP with an emphasis on the most recent developments pertaining to the function of these proteins.

PI-TP IN YEAST

PI-TP/SEC14p

A typical feature of mammalian PI-TP is its ability to mediate the transfer of PI and, to a lesser extent, that of PC between membranes. The first evidence that a protein with similar dual specificity occurred in yeast came from studies by Paltauf's group [17,18]. In a first attempt to elucidate the function, the gene encoding PI-TP (PIT1 gene) was isolated and disrupted by insertion of the LEU2 gene [8]. After transformation of the diploid strains with the *pil1::LEU2* gene, it was found that the spores containing the mutated gene were non-viable. These haploid strains could be ‘rescued’ by the presence of a plasmid-borne copy of the wild-type PIT1 gene, indicating that PI-TP was essential for cell viability. The *in vivo* function of PI-TP came more into focus by the discovery that the PIT1 gene was identical with the SEC14 gene [19]. By growing a temperature-sensitive *sec14-1^{ts}* strain at the permissive temperature, it was then possible to show that the membrane-free cytosol expressed PI-transfer activity when measured at 25 °C, but not at 37 °C [9]. In other words, at the ambient temperature of 37 °C the mutated PI-TP

Abbreviations used: PL-TP, phospholipid transfer protein; PC-TP, phosphatidylcholine transfer protein; nsL-TP, non-specific lipid transfer protein; CCTase, choline-phosphate cytidylyltransferase; ER, endoplasmic reticulum; SM, sphingomyelin; PKC, protein kinase C; PEP, priming in exocytosis protein; PIP-5-kinase, phosphatidylinositol-4-phosphate 5-kinase; PIP₂, phosphatidylinositol 4,5-bisphosphate; GTP[S], guanosine 5'-[γ -thio]-triphosphate; TGN, *trans*-Golgi network; EGF, epidermal growth factor; SCP₂, sterol carrier protein 2; CHO, Chinese-hamster ovary; PI(-TP), phosphatidylinositol (transfer protein); PLC, phospholipase C.

became inactive. This strongly suggested that the malfunction of the *sec14-1^{ts}* strain at the restrictive temperature was directly related to PI-TP not being able to transfer PI and/or PC *in situ*.

Regulation of PC biosynthesis

At this stage it is not known how yeast cell viability is related to another essential function of PI-TP (SEC14p), namely the maintenance of secretory-vesicle flow from the late-Golgi compartment [19,20]. By immunolocalization and subcellular fractionation it was shown that part of PI-TP is associated with the Golgi complex [21]. This raised the question whether the ability to transfer PI and PC has any significance for the role of PI-TP in vesicle flow. An unexpected twist to this question came from the observation that the dysfunction of PI-TP in the *sec14-1^{ts}* strain at the restrictive temperature could be by-passed by an additional mutation in the gene for yeast choline kinase, the first enzyme in the PC biosynthesis via the CDP-choline pathway [21]. PI-TP dysfunction was also overcome by a mutation in the gene for choline phosphotransferase, the ultimate enzyme in this pathway. Mutations in genes encoding enzymes associated with the methylation pathway for PC biosynthesis did not overcome the growth and secretory defects of the *sec14-1^{ts}* strain. These observations implied a specific connection between PI-TP function and the CDP-choline pathway [21]. As was shown by Bankaitis' group [22,23], the dual specificity of PI-TP with respect to PI and PC was a crucial factor in this connection.

From previous competition binding experiments using phospholipid vesicles of different PI/PC composition, it was estimated that bovine brain PI-TP has a 16-fold higher affinity for PI than for PC [24]. In that study, kinetic data provided evidence that, inherent to this characteristic difference in affinity, PI-TP will maintain a distinct PI/PC ratio in the membranes with which it interacts. A change in the membrane PI/PC ratio will be corrected by PI-TP by inserting PI in the membrane in exchange for PC, or vice versa. Kinetic studies with yeast PI-TP indicated that, *in vitro*, this protein behaves very similarly to bovine PI-TP [18]. Recent studies confirmed that indeed in yeast PI-TP may act as a sensor of the membrane PI/PC ratio, but then specifically of the Golgi complex [22,23]. Moreover, evidence was provided that PI-TP does not modulate the Golgi PI/PC ratio by the actual transfer of these phospholipids, but by the direct regulation of the CDP-choline pathway believed to be active in the Golgi. So it was demonstrated that the PI/PC ratio of Golgi isolated from a wild-type strain was 1.04, as compared with 0.39 for Golgi from *sec14-1^{ts}* mutants grown under restrictive conditions (37 °C) [22]. This shift in PI/PC ratio, which was specific for Golgi, was due to the fact that PI-TP decreased the rate of PC biosynthesis through the CDP-choline pathway in the wild-type and not in the mutant strain. In conclusion, the function of PI-TP is to maintain a reduced PC content in the wild-type Golgi membranes. There is no evidence that PI-TP has any effect on the PI levels of yeast Golgi membranes. To unravel further the connection with PC biosynthesis, PI-TP was overproduced in yeast strains deficient in any one of the three enzymes constituting the CDP-choline pathway, and the effect on the bulk PC was measured [23]. Similar to what was observed in wild-type cells, overproduction of PI-TP in these mutants gave a reduction in bulk PC, except in the strain deficient in choline-phosphate cytidyltransferase (CCTase), the rate-determining enzyme of the CDP-choline pathway. By *in vitro* assays it was then shown that yeast PI-TP specifically inhibited CCTase. This inhibition was species-specific, as rat PI-TP had no effect. Very interestingly, PI-TP acted as a molecular switch by only inhibiting

Scheme 1 Role of PI-TP in maintaining the PI/PC ratio of yeast Golgi membranes [22,23]

Interaction of PI-TP (SEC14p) with yeast Golgi membranes (light grey) gives PI-TP (dark pink) containing PI or PC. The relative amounts of the PI-TP(PC) and PI-TP(PI) form are determined by the levels of Golgi PI and PC. An increase of Golgi PC lowers the PI/PC ratio and, as a consequence, increases the relative amount of PI-TP(PC). This latter form inhibits CCTase, the rate-determining enzyme of PC synthesis, thereby restoring the initial PI/PC ratio. DG, diacylglycerol.

CCTase when it carried a PC molecule; PI-TP with PI was inactive.

From these data a model emerges in which PI-TP associated with the Golgi complex senses the PI/PC ratio of that very membrane (Scheme 1). Inherent to its exchange activity, PI and PC bound to PI-TP are in equilibrium with the Golgi PI and PC pool. An increase of the PC pool would increase the relative amount of PI-TP carrying a PC molecule, thereby inhibiting the CCTase activity so as to prevent a further increase of the Golgi PC pool relative to the PI pool. In this feed-back mechanism PI-TP acts as a ligand-modulated inhibitor to maintain the Golgi PI/PC ratio which is required for these membranes to function properly in the secretory pathway. At this stage it is not known how the actual budding of vesicles from the late Golgi is controlled by the strictly maintained PI/PC ratio. If the above model proves to be correct, it may mean that PC and other phospholipids that reach the Golgi by vesicle flow from the endoplasmic reticulum (ER) do not freely mix with the Golgi phospholipids. This may then even imply that vesicles flowing from the ER to the Golgi compartment participate in secretion by a 'kiss-and-run' mechanism.

PI-TP IN MAMMALS

PI-TP isoforms

In a recent study a rat brain cDNA library constructed on a yeast expression vector was screened for its ability to suppress the growth lesion of a mutant *sec14* strain at the restrictive temperature [25]. By this approach a rat cDNA clone was identified which 'rescued' this yeast mutant strain. Analysis of the nucleotide sequence showed that this cDNA encoded a protein the

amino acid sequence of which was 77% identical with (94% similar to) that of rat brain PI-TP [26]. As the cytosol from the rescued *sec14* strain expressed a distinct PI-transfer activity, it was concluded that this protein was an isoform of PI-TP. Hence, this isoform was designated PI-TP β , and the form initially purified from bovine brain was designated PI-TP α [2]. In independent studies a novel PL-TP was purified from chicken liver [27] and from bovine brain [28,29] which, by analogy to PI-TP α , could transfer PI and PC, but which also transferred sphingomyelin (SM) very efficiently. Quite unexpectedly, the first 21 N-terminal amino acid residues of this chicken and bovine protein were identical with those of rat PI-TP β , indicating that this novel PL-TP was PI-TP β .

The coding regions of rat and mouse PI-TP α cDNA encode 271 amino acid residues [26,30], whereas human and rabbit cDNA encode PI-TP α with a single chain of 270 residues [31,32]. This isoform has a molecular mass of 32.5 kDa and a pI of 5.5 or 5.7 depending on whether it contains a molecule of PI or PC [24]. Among species the primary structure of PI-TP α is highly conserved (98–99% identity). Similarly, the primary structures of rat and mouse PI-TP β consist of 271 residues and are 99% identical [25,33]. On the basis of the nucleotide sequence, yeast PI-TP (SEC14p) was found to consist of a single chain of 304 amino acid residues with a molecular mass of 35 kDa and a pI of 5.3 [8,19]. In addition to rat PI-TP β , growth and secretory defects of the *sec14-1^{ts}* strains were also overcome by the expression of rat PI-TP α [34]. This rescue is remarkable, since neither PI-TP isoform has any sequence homology with SEC14p. As for the rescue by PI-TP α , it was shown that this protein is not able to associate stably with yeast Golgi membranes [34] and is unable to inhibit yeast CCTase activity, the rate-limiting enzyme in PC biosynthesis [23]. As association with the Golgi and inhibition of PC synthesis are some of the key features of the model proposed by Bankaitis to explain the function of SEC14p, it remains to be established by what mechanism PI-TP α rescues the *sec14* mutants.

Intracellular localization

As shown by Western-blot analysis, antibodies raised against two synthetic peptides representing the predicted epitopes of rat brain PI-TP α were cross-reactive with both PI-TP α and PI-TP β [28,35]. On the other hand, antibodies raised against recombinant mouse PI-TP α were specific for this isoform [30]. By use of a combination of these two antibodies in indirect immunofluorescence studies on Swiss-mouse 3T3 fibroblasts, it was demonstrated that PI-TP α is localized in the cytoplasm and in the nucleus and that PI-TP β is preferentially associated with the Golgi system [28]. This distinct intracellular localization was further investigated by labelling bovine brain PI-TP α and PI-TP β each with a different sulphonylindocyanine dye (Cy3 and Cy5), followed by microinjection of a 1:1 mixture of these fluorescently labelled proteins into fetal-bovine heart endothelial cells [29]. By confocal laser scanning microscopy it was confirmed that Cy3-PI-TP α is predominantly distributed in the nucleus and the cytoplasm, and that Cy5-PI-TP β is preferentially associated with the Golgi system. However, this distinction was not absolute, as some PI-TP α was associated with the Golgi system and some PI-TP β localized in the nucleus and the cytoplasm. As shown by immunofluorescence microscopy, stimulation of quiescent Swiss-mouse 3T3 fibroblasts by PMA and bombesin gave a rapid (within 10 min) association of PI-TP with structures that represented the Golgi [36]. A similar stimulation of serum-starved semi-quiescent 3T3 fibroblasts resulted in a redistribution of PI-TP where, apart from the Golgi labelling, a distinct punctated

labelling was observed, possibly indicating the association of PI-TP with secretory vesicles (G. T. Snoek, personal communication). It still remains to be established whether one or both PI-TP isoforms are subject to this rapid redistribution. Given that both PMA and bombesin (by way of diacylglycerol) activate protein kinase C (PKC), one may assume that phosphorylation reactions directly affect the intracellular distribution of PI-TP. In this respect it should be noted that stimulation of semi-quiescent 3T3 cells by PMA significantly increased the phosphorylation of PI-TP [36]. Moreover, *in vitro*, PI-TP α was shown to be a substrate for PKC [28,36].

Role in vesicle flow

From the above studies it is evident that the association with the Golgi complex is a common feature of the PI-TP isoforms and SEC14p. It therefore made a lot of sense that, by analogy to SEC14p, PI-TP α and PI-TP β were identified as key components in the secretory pathway. In this identification the availability of permeabilized (semi-intact) cells and cell-free systems to assess the reconstitution of various steps of the secretion was of imminent importance. So it was shown, by using permeabilized cytosol-depleted PC12 cells, that the ATP-dependent priming of the Ca²⁺-activated secretion of noradrenaline (norepinephrine) required three priming-in-exocytosis-protein (PEP) factors. Upon isolation, one of these PEP factors, PEP3, was shown to be identical with PI-TP α [10,37]. PEP1 was later identified as a type I phosphatidylinositol-4-phosphate 5-kinase (PIP-5-kinase) [38]. Inhibition of the regulated secretion by phosphatidylinositol 4,5-bisphosphate (PIP₂)-specific antibodies implied that PIP₂ was involved in PI-TP/PIP-5-kinase-induced exocytosis. This, in conjunction with the requirement for ATP, led to the notion that, during priming, PI is phosphorylated to PIP₂ in consecutive steps by a resident PI-4-kinase present on the semi-intact cell membranes and the soluble type I PIP-5-kinase. In this model the function of PI-TP α is to carry PI to possibly distinct sites of phosphorylation, thereby promoting the synthesis of PIP₂. This same model was shown to hold for the guanosine 5'-[γ -thio]triphosphate (GTP[S])-stimulated Ca²⁺-activated secretion of hexosaminidase from permeabilized HL60 cells [39]. In the presence of ATP, protein secretion was restored by the addition of both PI-TP α and PI-TP β to the cytosol-depleted cells. Again this reconstitution was linked to a PI-TP-driven PIP₂ synthesis. In contrast with what was observed with the priming reaction in PC12 cells, PIP₂ synthesis in HL60 cells was dependent on GTP[S], implying the involvement of an as-yet-unidentified G-protein [39]. Very interestingly, PI-TP β was distinctly more active (about 5-fold) in restoring protein secretion than was PI-TP α . This may be the first evidence of a connection between the preferential association of PI-TP β with the Golgi system and its function in the secretory pathway. SEC14p also activated the ATP-dependent priming reaction [10]. We presume that its activity in this mammalian system is linked to the promotion of PIP₂ synthesis and not to the regulation of PC synthesis as observed in yeast (see above).

In the above studies the PI-TPs were instrumental in the fusion of secretory vesicles with the plasma membrane. Recently, however, these proteins were also identified as an essential factor in triggering vesicle budding and fission from the *trans*-Golgi network (TGN). In this identification use was made of a cell-free system derived from PC12 cells which consisted of highly purified *trans*-Golgi membranes carrying [³⁵S]heparan sulphate proteoglycan and [³⁵S]secretogranin II as marker proteins for the formation of constitutive secretory vesicles and immature secretory granules respectively. By using this assay, two active

proteins were purified from the cytosol of bovine adrenal medulla, CAST1 and CAST2 (cytosolic activity stimulating TGN vesicle formation) [11]. Very surprisingly, CAST1 was a mixture of PI-TP α and PI-TP β , confirming previous observations that the two isoforms are difficult to separate by regular chromatographic techniques [28]. As shown by using either bovine PI-TP α or PI-TP β , both isoforms were equally active in the budding reaction. The identity of CAST2 is not yet known, but given that the post-TGN secretory-vesicle formation required ATP, CAST2 may well turn out to be an inositol lipid kinase. If correct, the observed stimulation of vesicle budding may again be dependent on PI-TP-stimulated PIP₂ synthesis. In general, increased levels of PIP₂ could possibly lead to the recruitment of proteins at those membrane sites where vesicle budding and fission occur.

Role in inositol lipid signalling

Given that *de novo* synthesis of PI occurs on the ER and the receptor-controlled, PLC-mediated degradation of PIP₂ on the plasma membrane, it has long been proposed that PI-TP fulfils a role in this process by delivering PI to sites of phosphorylation present on the plasma membrane as well [40–42]. That indeed PI-TP is an essential factor in inositol lipid signalling was convincingly demonstrated by Cockcroft's group using permeabilized HL60 leucocytes and A431 epidermoid carcinoma cells [12,43,44]. In those studies, prior to permeabilization, the cells were incubated with [³H]inositol for 48 h to equilibrium-label the total phosphoinositide pool. After permeabilization with streptolysin O, the production of total [³H]inositol phosphates (IP_n) was taken as a measure of inositol-lipid signalling. So it was shown that cytosol depletion greatly diminished the GTP[S]-mediated PLC β activity of the HL60 cells, but that this activity could be reconstituted by the addition of cytosolic components from bovine brain. Purification then led to the identification of PI-TP α as the active component in bovine brain cytosol [12]. Under these conditions, bovine PI-TP β and SEC14p were equally effective in reconstituting G-protein-mediated PLC β activity [28,45]. In a comparable study, permeabilization of A431 cells led to a complete leakage of PI-TP and epidermal-growth-factor (EGF)-receptor-controlled PLC γ into the extracellular medium [44]. Activation of these cytosol-depleted cells by EGF in the presence of either PI-TP or PLC γ gave a minimal production of IP_n. However, IP_n production became highly elevated when PI-TP and PLC γ were both present. This indicated that the expression of the EGF-receptor-controlled PLC γ activity was dependent on the presence of PI-TP. This protein was also required for the reconstitution of GTP[S]-mediated PLC β - and of tyrosine kinase-controlled PLC γ activity in cytosol-depleted rat basophilic leukaemia (RBL-2H3) cells [45]. In the latter study, PI-TP α , PI-TP β and SEC14p were equally effective, strongly suggesting that the ability shared by all three proteins to bind and transfer PI was sufficient to restore inositol lipid signalling.

All available data indicate that the link between PI-TP and restoration of PLC activity bears on the ability of PI-TP to stimulate the synthesis of PIP₂, in particular that pool which serves as a substrate for receptor-controlled PLC [43,44]. In support of this, the addition of PI-TP to permeabilized A431 cells stimulated PIP₂ synthesis provided that EGF was present. Apparently, PI-4/PIP-5-kinase activity is regulated by EGF, but requires PI-TP for full implementation [44]. In the permeabilized HL60 cells the PI-TP-controlled, GTP[S]-mediated PLC β activity yielded inositol triphosphate far in excess of the amount of PIP₂ present [43]. In that study GTP[S] was shown to stimulate PIP₂ synthesis, yet PI-TP was required for this synthesis to become fully expressed. In summary, these studies may indicate that

Scheme 2 PI-TP-induced vesicle flow provides the caveolae with PIP₂ which serves as substrate for GTP[S]-mediated phospholipase C

Triggering of vesicle budding from the *trans*-Golgi network (light grey) and of vesicle fusion with the plasma membrane by PI-TP (dark pink) is accompanied by increased PIP₂ synthesis. Vesicles (dark grey) enriched in PIP₂ are delivered to the caveolae (light pink), where receptor-controlled PLC converts PIP₂ into diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP₃).

receptor-activation triggers the assembly of a signal-producing complex consisting of inositol lipid kinases and PLCs, with which PI-TP interacts to deliver PI for phosphorylation. In this context it has been proposed that, during phosphorylation, PI, PIP and PIP₂ remain bound to PI-TP for PIP₂ then to be degraded by PLC [43]. At this stage it is generally assumed that this phosphorylation cascade and PIP₂ degradation occur on the plasma membrane. This model, however, is hard to reconcile with the observation that PI-TP has no tendency to collect at, or in any way be associated with, the plasma membrane [29,36]. Rather this suggests the possibility that, in permeabilized cells, the activation of GTP[S]-mediated PLC activity by PI-TP is a reflection of GTP[S]-stimulated protein secretion, which proceeds by PI-TP stimulating PIP₂ synthesis [39]. As shown by the model in Scheme 2, the association of PI-TP with the TGN and secretory vesicles is presumed to be required for this protein to induce vesicle fission and fusion [10,11]. Induction of these processes is linked to PI-TP stimulating PIP₂ synthesis, possibly at the sites of budding and/or in the vesicles primed for secretion [11,38]. These vesicles enriched in PIP₂ are targeted to sites on the plasma membrane where the GTP[S]-controlled PLC expresses its activity. These sites may well be the caveolae, small plasma-membrane invaginations to which the hormone-sensitive PIP₂ pool appears to be restricted [45a]. By way of this vesicle flow, caveolae are also supplied with PI and PIP. It still remains to be determined what specific role PI-TP α and PI-TP β play in this vesicle flow–lipid signalling model.

NON-SPECIFIC LIPID-TRANSFER PROTEIN IN MAMMALS

Lipid binding and transport

Apart from PI-TP, mammalian tissues contain another protein that, *in vitro*, stimulates the transfer of PI between membranes. This protein, however, initially purified from the membrane-free supernatant of rat and bovine liver, was non-specific, being able to mediate the transfer of all common phospholipids, but also that of cholesterol [3,46]. The ability to shuttle cholesterol and other sterols between membranes had the interesting effect that, *in vitro*, this nsL-TP stimulated the microsomal conversion of lanosterol into cholesterol, cholesterol esterification, bile acid formation and the mitochondrial conversion of cholesterol into

pregnenolone (for reviews, see [13,47]). Because of this apparent involvement in cholesterol metabolism, nsL-TP is also denoted SCP₂. However, contrary to what the name implies, there is very little evidence that, *in vitro* and upon interaction with a membrane, SCP₂ binds cholesterol and actually forms a stable complex to the extent that cholesterol is carried through the aqueous phase between membranes [14,48]. On the other hand, nsL-TP has a lipid binding site which, upon interaction with membranes, can accommodate a phospholipid molecule [49,50]. Moreover, nsL-TP can bind fatty acids and long-chain fatty-acyl-CoAs [51]. Because of its low affinity, the binding site is occupied by lipid only as long as the membranes are present [52]. In this sense nsL-TP differs fundamentally from PI-TP, which forms a stable complex with phospholipids (see above). Given that this lipid binding site appears to lack clear conformational constraints, it can be expected that, *in vitro* and under certain conditions, cholesterol will bind to nsL-TP [53,54]. So far, studies on intact cells have failed to provide convincing evidence that nsL-TP is involved in intracellular cholesterol or phospholipid transport [55,56]. As for its involvement in cholesterol metabolism, nsL-TP does play a role in regulating steroidogenesis, yet the mechanism by which remains to be elucidated [57,58]. Be that as it may, molecular characterization has indicated that nsL-TP belongs to a new and, most likely, very important class of proteins.

Molecular characterization

The primary structure of nsL-TP deduced from the encoding cDNAs consists of a single chain of 123 residues with an amino-acid-sequence identity between species (rat, mouse and human) of more than 90% [58–60]. The C-terminal end consists of the tripeptide Ala-Lys-Leu, which is a peroxisomal targeting sequence [61]. An interesting point is that the primary sequence of nsL-TP purified from bovine liver was found to lack the C-terminal Lys-Leu [62], and nsL-TP from rat liver lacks the C-terminal Leu [63]. These modifications may have implications for the intracellular localization of nsL-TP (see below). Analysis of the cDNAs revealed that all nsL-TPs are expressed in a precursor form carrying a highly conserved 20-residue N-terminal leader sequence. This sequence has some resemblance with a mitochondrial targeting presequence [60]. The amino acid sequence of nsL-TP (molecular mass of 13.2 kDa; pI 8.6–9.6) has as interesting features a single tryptophan residue at position 50, a single cysteine residue at position 71 and the absence of arginine, histidine and tyrosine residues. The single tryptophan residue makes nsL-TP very suitable for probing the lipid binding site by time-resolved and steady-state fluorescence spectroscopy [50,54]. Blockage of the single cysteine residue by *N*-ethylmaleimide inactivated the protein [64,65]. Dimerization of nsL-TP by the single cysteine residue was proposed as a mechanism for two bilayers to associate closely so as to enable lipid molecules to move between membranes [14,64].

By Northern-blot analysis of polyadenylated RNA, four nsL-TP related mRNAs (1.1, 1.7, 2.4 and 3.0 kb) were detected in rat and mouse liver [66–68] and two mRNAs (1.8 and 3.2 kb) in human liver [58]. The 1.7 and 3.0 kb mRNA species were generated from the 1.1 and 2.4 kb mRNA by alternative polyadenylation [68]. These mRNAs derive from the cDNAs encoding the 15.3 kDa precursor of nsL-TP (pre-nsL-TP) and a 58 kDa protein, initially identified in rat liver by Western blotting using an anti-nsL-TP antibody [69]. Analysis of the cDNA encoding the 58 kDa protein (also denoted SCP_x) revealed that the protein consisted of a 404-amino-acid N-terminal domain and a 143-amino-acid C-terminal domain that was identical with pre-nsL-TP [66–68,70–72]. Recently, it was shown that pre-nsL-TP and

the 58 kDa protein were expressed from a single gene (SCP_x/SCP₂ gene) by alternative transcription initiation from two distinct promoters [73].

Localization in peroxisomes

By immunoelectron-microscopic labelling of rat liver sections using an affinity-purified antibody against nsL-TP, labelling was found to be highly concentrated in the matrices of the peroxisomes [69,74,75]. Some low-intensity labelling was observed inside the mitochondria, on the ER and in the cytosol. A predominantly peroxisomal labelling was also observed with rat Leydig cells [76], rat lung type II cells [77] and with rat small-intestine enterocytes [78]. Since the antibody was cross-reactive with both nsL-TP and the 58 kDa protein, the observed peroxisomal labelling may be due to either protein. Immunoblot analysis of isolated subcellular fractions using the anti-nsL-TP antibody confirmed that nsL-TP and the 58 kDa protein were present in the peroxisomes [69,74,75]. In addition, nsL-TP was found in the membrane-free cytosol fraction. Owing to the fragility of the peroxisomes, part of the cytosolic nsL-TP may have leaked out of these organelles during homogenization. Moreover, nsL-TPs without an intact peroxisomal targeting sequence have been detected in bovine and rat liver cytosol, possibly reflecting post-translational processing [62,63].

With nsL-TP and the 58 kDa protein predominantly present in peroxisomes, studies were carried out to determine what would happen to these proteins in cells that lack peroxisomes [56,79,80]. For these studies, cells were used derived from patients with the Zellweger syndrome [81] and peroxisome-deficient Chinese-hamster ovary (CHO) cells [82]. As shown by immunoblot analysis, the liver from the Zellweger patients and the mutant CHO cells completely lacked nsL-TP, whereas the 58 kDa protein was still present. This suggested that, in the absence of peroxisomes, nsL-TP is susceptible to rapid degradation. In line with these results immunofluorescence staining of fibroblasts from Zellweger patients using the anti-nsL-TP antibody did not give the characteristic punctated labelling of peroxisomes as observed in control fibroblasts [80].

Peroxisomal processing

Cell-free translation using total RNA of rat liver in combination with pulse-chase experiments in rat hepatoma H-35 cells and in human fibroblasts have indicated that nsL-TP is synthesized as a precursor of 14.5 kDa (pre-nsL-TP) on cytoplasmic free polyribosomes. After synthesis, pre-nsL-TP is transported to peroxisomes, where it is proteolytically processed to the mature form of 13 kDa [80,83]. Pulse-chase experiments in fibroblasts of Zellweger patients demonstrated that pre-nsL-TP was normally synthesized and then rapidly degraded [80]. An interesting point is that, in addition to a C-terminal peroxisomal targeting signal, pre-nsL-TP has a 20-amino-acid presequence which supposedly is cleaved by a peroxisomal protease [84]. We do know that this C-terminal tripeptide is essential for targeting, as pre-nsL-TP lacking the C-terminal leucine residue did not accumulate in the peroxisomes (F. S. Wouters, P. I. H. Bastiaens, K. W. A. Wirtz and T. M. Jovin, unpublished work). At this point it is not clear whether the presequence of pre-nsL-TP serves any function in the peroxisomal targeting. In this respect, one is reminded that the 58 kDa protein, which is also targeted towards the peroxisomes, contains pre-nsL-TP at its C-terminal end (Figure 1). By immunoblotting of rat liver fractions using antibodies raised against peptide regions of the N-terminal part of the 58 kDa protein, the 58 kDa protein and a cross-reactive 46 kDa protein

Figure 1 Relationship between nsL-TP (SCP_2) and the 58 kDa protein (SCP_x)

Pre-nsL-TP has a peroxisomal targeting signal (AKL) and a presequence of 20 amino acid residues (light grey) which is cleaved off upon import into peroxisomes. The 58 kDa protein identified as a peroxisomal 3-oxoacyl-CoA thiolase contains pre-nsL-TP as an N-terminal domain. It is proposed that the peroxisomal 46 kDa protein originates from the 58 kDa protein. AA, amino acids.

Scheme 3 Transcription/translation of the SCP_x/SCP_2 gene (dark pink) yielding the 58 kDa protein and pre-nsL-TP

Import of the 58 kDa protein and pre-nsL-TP into different subpopulations of peroxisomes (light pink) accompanied by processing results in a heterogeneous distribution of nsL-TP.

were detected [85]. This 46 kDa protein was prominently present in liver and adrenals, but was also detected in other rat tissues. As shown by immunogold labelling, the 46 kDa protein was located in the peroxisomes. This led to the proposal that, in peroxisomes, the 58 kDa protein can be processed to the 46 kDa protein and nsL-TP, supposedly by the same protease that cleaves pre-nsL-TP [85]. This would imply that, in peroxisomes, nsL-TP may derive from two precursor proteins (Scheme 3).

Given the heterogeneous distribution of enzymes in peroxisomes, it is still possible that in one population of peroxisomes nsL-TP derives from pre-nsL-TP and in a second population from the 58 kDa protein [86].

Function in peroxisomes

Peroxisomes play an important role in lipid metabolism, including the β -oxidation of very-long-chain and branched-chain fatty acids and the synthesis of cholesterol, bile acids, ether-linked phospholipids and dolichol (reviewed in [87]). Similar to the *in vitro* stimulation of microsomal cholesterol synthesis [88,89], bile acid formation [90] and dolichol synthesis [91] by nsL-TP, one should consider the possibility that these very same reactions are stimulated by nsL-TP in peroxisomes. Apart from these possible functions, there is convincing evidence that, in peroxisomes, nsL-TP interacts directly with enzymes involved in the β -oxidation of fatty acids (F. S. Wouters, P. I. H. Bastiaens, K. W. A. Wirtz and T. M. Jovin, unpublished work). Given that nsL-TP binds fatty acids as well, it is very likely that this protein delivers substrates to the β -oxidation pathway. A similar function was proposed for the analogue of nsL-TP in yeast [92]. Enzymes of β -oxidation are also involved in the peroxisomal conversion of trihydroxy-5 β -cholestanoic acid into cholic acid [93]. This explains the accumulation of bile acid intermediates in Zellweger syndrome. It is possible that nsL-TP is involved in this side-chain cleavage reaction as well. In mice carrying a null mutation for the SCP_x/SCP_2 gene, the serum concentration of phytanic acid was significantly increased, indicating an inefficient peroxisomal oxidation of this branched-chain fatty acid, which is a metabolite of phytol from the diet (U. Sedorf, M. Rabbe, M. Fobker, T. Engel, F. Kannenberg, S. Denis, F. Wouters, K. W. A. Wirtz, R. J. A. Wanders, N. Maeda and G. Assmann, unpublished work). Feeding of a diet enriched in phytol to these knock-out mice led to premature death. Since the SCP_x/SCP_2 gene encodes both nsL-TP and the 58 kDa protein [73], it still remains to be established whether one or either protein is involved in this oxidation process.

From comparison with the GenBank/EMBL database it was

noted that the sequence of the first 400 residues of the rat liver 58 kDa protein (the putative 46 kDa protein) was 50 % similar to those of the peroxisomal and mitochondrial 3-oxoacyl-CoA thiolases [67]. Recombinant 58 kDa protein (also denoted SCP_s) obtained after expression of the encoding gene in *Escherichia coli* indeed cleaved 3-oxo-acyl-CoA [16]. In addition, *in vitro* this novel peroxisomal thiolase expressed nsL-TP activity. This intrinsic transfer activity may be essential for delivering the substrate to the active site. At this point it is not known whether the putative 46 kDa protein has thiolase activity (see Scheme 3). Recently, the porcine 17 β -hydroxysteroid dehydrogenase type IV (80 kDa) was identified as another multidomain peroxisomal protein the C-terminal part of which being similar to nsL-TP [94]. By expression of the encoding cDNAs in *Escherichia coli*, the N-terminal peptide (amino acids 1–323) was shown to have the dehydrogenase activity, the central part (amino acids 324–596) the 2-enoyl-acyl-CoA hydratase activity, and the C-terminal peptide (amino acids 597–737) the nsL-TP activity [95]. It is remarkable that the 58 kDa and 80 kDa proteins have a multidomain structure the composition of which is related to enzymes involved in the peroxisomal β -oxidation of fatty acids. The specific function of the nsL-TP-segment in these enzymes remains to be established.

CONCLUDING REMARKS

All available evidence indicates that both PI-TP and nsL-TP are non-enzymic proteins which through their ability to interact with certain lipids play an important role in lipid metabolism and, thereby, regulate essential cellular processes. It is to be noted that the effect of PI-TP on lipid metabolism in yeast appears to be fundamentally different from that in mammals. Since in both systems PI-TP is an essential component of the secretory pathway, further studies have to be awaited to see whether these differences can be resolved. The stimulatory effect of PI-TP on PIP₂ synthesis in mammalian cells appears to be essential both for PLC-linked signal transduction and for secretory-vesicle flow. A point still to be resolved is whether PI-TP is indeed directly involved in these two processes or whether, by stimulating PIP₂ synthesis, PI-TP establishes a link between these two processes (see Scheme 2). This review has focused mainly on some of the possible functions of PI-TP and nsL-TP. Other PL-TPs have been, or still may be, identified in the many species and organisms awaiting to be explored. From the picture that has emerged in the last few years it is fair to state that the investigation of the PL-TPs has just begun and is bound to provide us with many exciting and novel insights in their role as regulators of phospholipid metabolism in connection to specific cellular functions.

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