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Molecular Analysis of Lipid Synthesis and Its Role in Vesicle Trafficking: *De novo* Cloning, Expression, and Characterization of Two Human Cholinephosphotransferases

by

Annette Lisa Henneberry

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Dalhousie University

at

Department of Biochemistry & Molecular Biology
Dalhousie University
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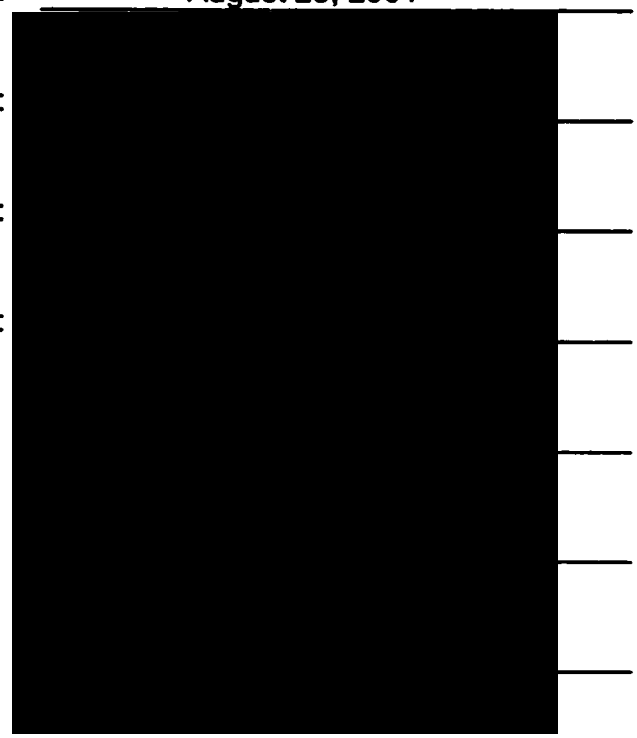

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For my parents, Brenda and Earl,

whose support for me has never wavered in 27 years

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Abstract

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the most abundant phospholipids in eukaryotic cells, comprising 50% and 25% of membrane phospholipid mass, respectively. Cholinephosphotransferase catalyzes the final step in the *de novo* biosynthesis of PC through the Kennedy pathway *via* the transfer of phosphocholine from CDP-choline to diacylglycerol, with the release of CMP. Ethanolaminephosphotransferase catalyzes an analogous reaction with CDP-ethanolamine as the phosphobase donor for the *de novo* biosynthesis of PE. Together, these two enzyme activities determine both the site of synthesis and the fatty acyl composition of PC and PE synthesized *de novo*. Attempts to purify these enzymes have been largely unsuccessful due to their integral membrane bound nature, and thus, most of the studies to date have used microsomal membrane preparations to characterize these enzymes. The recent cloning of the *Saccharomyces cerevisiae* choline- and ethanolaminephosphotransferases (*CPT1* and *EPT1*) has facilitated the search for mammalian cDNAs encoding these enzyme activities. This work reports the cloning, expression, and characterization of the first mammalian choline- and ethanolaminephosphotransferase cDNAs. Human choline/ethanolamine-phosphotransferase 1 (hCEPT1) codes for a 416 amino acid protein that can catalyze the *de novo* biosynthesis of both PC and PE *in vitro* and *in vivo*. Human cholinephosphotransferase 1 (hCPT1) encodes a 406 amino acid protein specific for the enzymatic synthesis of PC *in vitro* and *in vivo*. *In vitro* assessment of hCEPT1 and hCPT1-derived cholinephosphotransferase activity revealed differences in diradylglycerol specificities including their capacity to synthesize platelet-activating factor and platelet-activating factor precursor. While hCEPT1 mRNA was found in similar abundance in all tissues tested, expression of hCPT1 mRNA varied greater than 100-fold between tissues, and was most abundant in testis, followed by colon, small intestine, heart, prostate, and spleen. Immunofluorescence studies revealed that hCEPT1p was localized to the ER and the nuclear membrane, while hCPT1p was localized to the Golgi apparatus. Site-directed mutagenesis of hCEPT1 demonstrated that glycine^{156} plays a role in CDP-ethanolamine binding, while threonine^{214}, valine^{216}, and isoleucine^{221} appear to play a role in determining the specificity of DAG utilized by hCEPT1p. Mutation of glutamate^{215} suggested that it might play a role in both DAG and CDP-aminoalcohol binding, although the exact roles remain to be elucidated.

This work has also investigated the role of PC in the diacylglycerol homeostasis required for Sec14p-dependent Golgi function and cell growth in yeast. We have shown that, in contrast to previous work, *CPT1*- and *EPT1*-derived cholinephosphotransferase activities can significantly overlap *in vivo* such that *EPT1* can contribute 60% of net PC synthesis *via* the Kennedy pathway. Variation in the level of diacylglycerol consumption through alterations in PC synthesis directly correlated with the level of *SEC14*-dependent invertase secretion and affected cell viability. Administration of synthetic di8:0 diacylglycerol resulted in a partial rescue of cells from sec14-mediated cell death. The addition of di8:0 diacylglycerol did not alter endogenous phospholipid metabolic pathways, nor was it converted to di8:0 phosphatidic acid. In addition, only hCEPT1-derived activity, and not hCPT1, was able to complement the yeast *CPT1* gene in its interaction with *SEC14* and affect cell growth.

List of Abbreviations

AAPT	aminoalcoholphosphotransferase
BFA	brefeldin A
BSA	bovine serum albumin
CDP-choline	cytidine 5'-diphosphocholine
CDP-ethanolamine	cytidine 5'-diphosphoethanolamine
CEPT	choline/ethanolaminephosphotransferase
CHO	Chinese hamster ovary
CK	choline kinase
cpm	counts per minute
CPT	*sn*-1,2-diacylglycerol cholinephosphotransferase
CPT1	yeast cholinephosphotransferase gene
CT	CTP:phosphocholine cytidylyltransferase
DAG	*sn*-1,2-diacylglycerol
dH_2O	distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethylsulfoxide
dNTP	dideoxynucleotide triphosphate
dpm	disintegrations per minute
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylene-diaminetetra-acetic acid

EK	ethanolamine kinase
EPT	*sn*-1,2-diacylglycerol ethanolaminephosphotransferase
EPT1	yeast choline/ethanolaminephosphotransferase gene
ER	endoplasmic reticulum
ET	CTP:phosphoethanolamine cytidylyltransferase
FBS	fetal bovine serum
FITC	fluorescein-5-isothiocyanate
GST	glutathione *S*-transferase
IPTG	isopropyl-1-thio-β-D-galactopyranoside
kbp	kilobase pairs
kDa	kilodalton
LB	Luria-Bertani medium
M	molar
mM	millimolar
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
PA	phosphatidic acid
PAF	platelet-activating factor
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PC	phosphatidylcholine
PCho	phosphocholine
PCR	polymerase chain reaction

PE	phosphatidylethanolamine
PEMT	phosphatidylethanolamine *N*-methyltransferase
PEtn	phosphoethanolamine
PI	phosphatidylinositol
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PLMT	phospholipid methyltransferase
PM	plasma membrane
PS	phosphatidylserine
PVDF	polyvinylidene fluoride
SD	synthetic dextrose medium
SDS	sodium dodecyl sulfate
SM	sphingomyelin
SMS	sphingomyelin synthase
TBS	Tris-buffered saline
TE	Tris/EDTA buffer
TLC	thin-layered chromatography
Tris	Tris (hydroxymethyl)aminomethane
v/v	volume per volume
w/v	weight per volume
YEPD	yeast extract peptone dextrose medium

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I. Introduction

There have been two distinct phases in the history of lipid biochemistry. For many decades, the study of lipid synthesis and its regulation has been facilitated by partial purification of enzymes, analysis of subcellular preparations, and metabolic analysis. More recently, with the sequencing of a number of genomes, these analyses have been supplemented by molecular biological and genetic techniques. Specifically, this involves the cloning, expression, and characterization of various proteins and enzymes with roles in lipid synthesis. Biological membranes are complex, with over 1000 distinct chemical species of membrane lipids known to occur in eukaryotic cells (Raetz, 1986). Therefore, identifying the proteins and enzymes involved in their synthesis and regulation is an important step in understanding the complex nature of lipids.

A. Phosphatidylcholine

Phosphatidylcholine (PC) is the major phospholipid present in eukaryotic cells, comprising approximately 50% of cellular phospholipid mass (Paltauf et al., 1992; Raetz, 1986). In contrast, most prokaryotes do not contain PC as a membrane lipid. Gobley first discovered PC in 1845 as a component of egg yolk and named it lecithin in 1850 after lekithos, the Greek term for egg yolk. The pathway for the *de novo* biosynthesis of PC, like many other phospholipids, was elucidated by Eugene Kennedy in 1956.

1. Structure and biosynthesis

PC is comprised of a glycerol backbone with fatty acids esterified at the *sn*-1 and *sn*-2 positions. Generally, the fatty acid at the *sn*-1 position is more saturated than the fatty acid at the *sn*-2 position, which is commonly arachidonic acid in PC. The third position on the glycerol backbone is occupied by a phosphate group to which the polar head

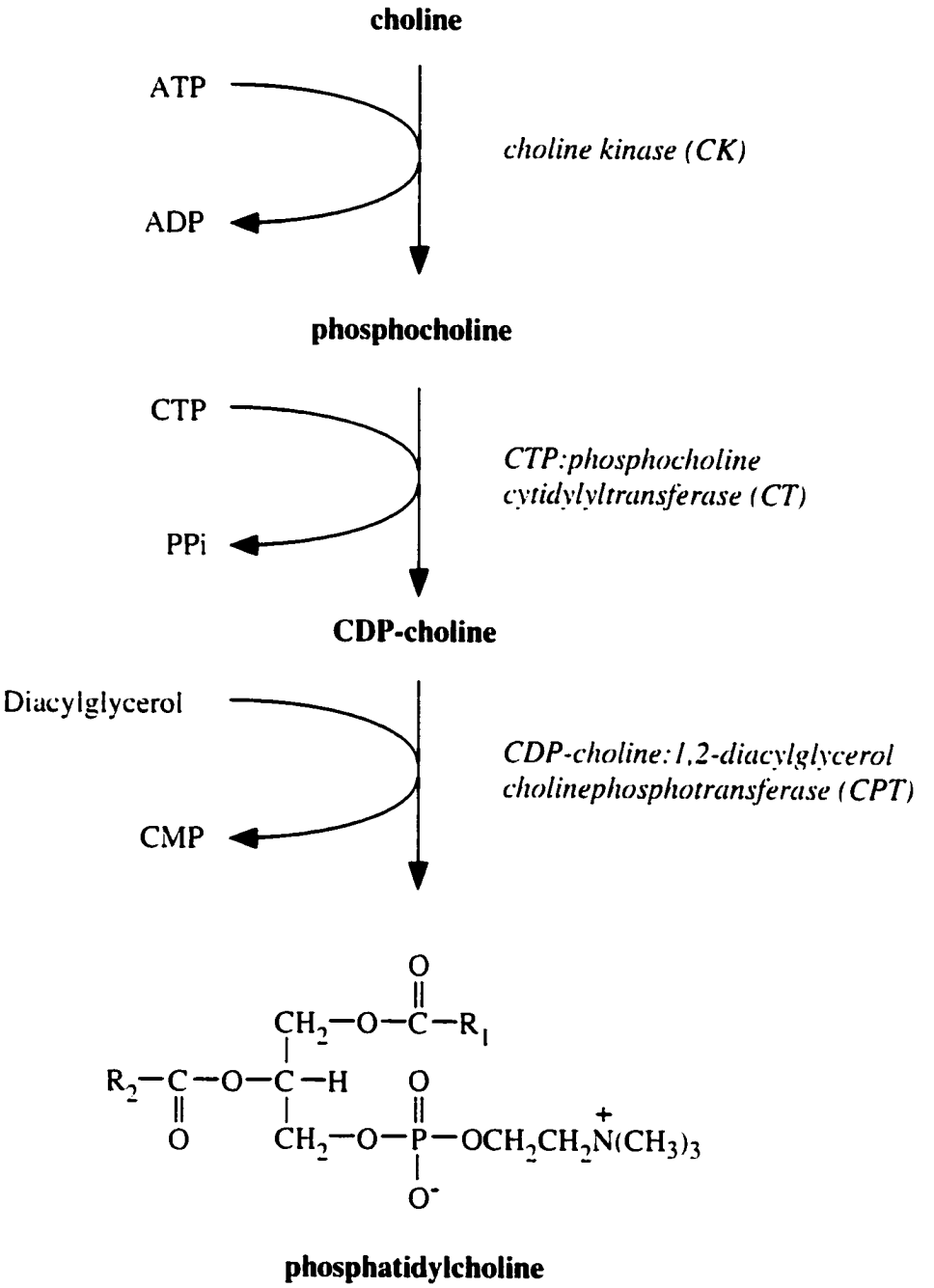


Figure 1. CDP-choline pathway for *de novo* PC biosynthesis. R_1 and R_2 denote long-chain fatty acids.

group, choline, is attached by a phosphodiester bond (Figure 1). There are a number of ways by which cells can synthesize PC. In mammalian cells, the most predominant method is through the Kennedy, or CDP-choline, pathway (Kennedy, 1956). As shown in Figure 1, upon the import of choline into the cell *via* a choline transporter, it is phosphorylated by choline kinase to synthesize phosphocholine. Next, CDP-choline is synthesized from phosphocholine and cytidine triphosphate (CTP), releasing pyrophosphate (PP_i). This rate-limiting step is catalyzed by CTP:phosphocholine cytidylyltransferase (CT). The final step in the pathway is catalyzed by cholinephosphotransferase (CPT) and involves the transfer of phosphocholine from CDP-choline to diacylglycerol to produce PC, releasing cytidine monophosphate (CMP). The enzymes catalyzing each reaction are described in more detail in the following sections.

Yeast and mammalian liver cells can also synthesize PC through three successive methylations of phosphatidylethanolamine (PE). In yeast cells, these reactions are catalyzed by two phospholipid *N*-methyltransferases (PLMTs), both of which use *S*-adenosyl-methionine (SAM) as a methyl donor. These two PLMTs are commonly referred to as class I and class II PLMTs and the genes encoding both classes have been cloned from both budding yeast, *Saccharomyces cerevisiae*, and fission yeast, *Schizosaccharomyces pombe*. The class II enzymes catalyze the first methylation step from PE to phosphatidylmonomethylethanolamine (PMME) and the gene encoding the enzyme was cloned separately by two groups by complementing *pem1* (Kodaki and Yamashita, 1987) and *cho2* (Summers et al., 1988) mutations in yeast. The *PEM1/CHO2* gene encodes an 869-amino acid protein whose hydropathy profile suggests that it is an integral membrane protein. The class I enzymes catalyze the second (PMME to

phosphatidyldimethylethanolamine (PDME)) and third (PDME to PC) methylation reactions in the formation of PC in yeast. Similar to the class II enzyme, the gene encoding the class I enzyme was cloned independently by two groups by complementation of the *pem2* (Kodaki and Yamashita, 1987) and *opi3* (McGraw and Henry, 1989) mutations in yeast. Interestingly, it was also cloned as an *in vivo* high-copy suppressor of the *cho2* mutant, implying its substrate specificity may extend to include PE (Preitschopf et al., 1993). The *PEM2/OPI3* gene encodes a 206-amino acid protein that is also predicted to be an integral membrane protein. Although neither yeast enzyme has been purified, subcellular fractionation studies have localized both classes of PLMTs to the endoplasmic reticulum (Zinser et al., 1991).

Unlike yeast, mammalian cells contain only one enzyme for methylating PE. Most of the phosphatidylethanolamine *N*-methyltransferase (PEMT) activity is associated with the endoplasmic reticulum (PEMT1), although a second isoform of the enzyme has recently been localized to the mitochondria-associated membrane (PEMT2) (Cui et al., 1993). PEMT was first purified in 1987 from rat liver and was found to consist of a single subunit with an apparent molecular mass of 18.3 kDa (Ridgway and Vance, 1987). The single, low molecular weight protein was capable of converting PE to PC, although earlier reports had suggested that two enzymes were involved in this process (Vance and Ridgway, 1988). Using the N-terminal sequence determined from the purified PEMT, the cDNA encoding PEMT2 was isolated from a rat liver cDNA library (Cui et al., 1993). It was found to encode a protein of 199 amino acids with a predicted molecular mass of 22.3 kDa, which was 44% identical to the yeast *PEM2* gene product. Similar to *PEM2*, the cloned PEMT2 was able to methylate PE, PMME, and PDME for the synthesis of PC

(Cui et al., 1993). A polyclonal antibody to a synthetic carboxyl-terminal peptide of PEMT2 recognized only a protein on the mitochondrial-associated membrane (MAM), distinguishing it from PEMT1, which is located in the ER. Although the two isoforms have different subcellular locations, a single gene encodes both activities as demonstrated by the cloning and targeted disruption of the murine gene for PEMT2 (Walkey et al., 1996; Walkey et al., 1997). When the *Pempt* gene was disrupted in mice, there was no detectable PEMT activity remaining, indicating that both isoforms of PEMT are transcribed from the same gene. In addition, the mice displayed no abnormal phenotype, normal hepatocyte morphology, normal plasma lipid levels, and no differences in bile composition, and thus the *Pempt* gene is not required for normal growth and reproduction (Walkey et al., 1997). However, the absence of dietary choline in mice lacking PEMT causes severe liver pathology. Therefore, PEMT appears to be required to maintain hepatic PC biosynthesis when the CDP-choline pathway is insufficient, such as might occur during starvation or pregnancy (Walkey et al., 1998).

Another potential function for PEMT has been suggested by correlations between hepatocyte growth rates and PEMT2 expression. Proliferating hepatocytes express lower levels of PEMT2 compared to non-proliferating hepatocytes and these results have suggested that PEMT2 has a role in regulating hepatocyte growth and liver cancer (Cui et al., 1997; Pelech et al., 1983; Sesca et al., 1996). In addition, PEMT2 expression is inhibited when hepatocyte proliferation is induced by partial hepatectomy (Houweling et al., 1997) and, when tumorigenesis is chemically induced in rat livers, PEMT2 expression is decreased early in the hepatocarcinogenesis process (Cui et al., 1994; Tessitore et al., 1999). Other studies have demonstrated that although the end product of both the CDP-

choline and PEMT pathways is PC, the two pathways are functionally different and not interchangeable (Houweling et al., 1995). One hypothesis for this distinction is that the two pathways synthesize different pools of PC molecular species. This hypothesis was supported by the finding that the PC species derived from the CDP-choline pathway were mainly comprised of medium-chain and saturated species, whereas the PC species synthesized from the methylation pathway were mainly comprised of long-chain and highly unsaturated species (DeLong et al., 1999). Although a number of hypotheses regarding the methylation pathway have been put forth, its functional significance is not completely understood.

The only other known pathway for synthesizing PC occurs in prokaryotes, *via* the condensation of CDP-diacylglyceride and choline, in a one-step reaction catalyzed by phosphatidylcholine synthase. Until recently, it was believed that only the methylation pathway occurred in prokaryotes. However, in the soil bacterium *Sinorhizobium (Rhizobium) meliloti*, a mutant with a defective methylation pathway was still able to make PC, suggesting that a second pathway for PC biosynthesis occurs in certain prokaryotes (de Rudder et al., 1997). This observation led to the cloning and characterization of the gene for phosphatidylcholine synthase in *S. meliloti* (de Rudder et al., 1999; Sohlenkamp et al., 2000). Similarity searches using the BLAST algorithm revealed open reading frames with high amino acid identity to PC synthase in *Rhodobacter capsulatus* (43%), *Pseudomonas aeruginosa* (39%), and *Borrelia burgdorferi* (29%).

a. Choline kinase (CK)

Choline kinase (CK) catalyzes the phosphorylation of choline as the initial step in the *de novo* biosynthesis of PC through the Kennedy pathway. The activity was first reported in brewer's yeast when it was discovered that phosphorylcholine was incorporated into phospholipid at a much higher rate than was free choline *in vitro* (Wittenberg and Kornberg, 1953). CK is a soluble enzyme that has been localized to the cytosol, except in a few instances where its activity has been detected in synaptosomes (Reinhardt et al., 1984) and highly purified myelin fractions (Kunishita et al., 1987) from rat brain. There have been a number of successful purification attempts from rat kidney (Ishidate et al., 1984), liver (Porter and Kent, 1990), and brain (Uchida and Yamashita, 1990). Although all of the purified preparations showed high CK activity, all have been shown to possess significant ethanolamine kinase (EK) activity as well, suggesting that the two activities reside on the same protein. In contrast, there have been reports that suggest the existence of a separate EK enzyme (Brophy et al., 1977; Weinhold and Rethy, 1974), which is discussed further in section B.1 (a).

The gene encoding CK, *CKI1*, was first cloned from *S. cerevisiae* in 1989 by complementation of *cki* mutants (Hosaka et al., 1989). The *CKI1* gene encodes a protein of 582 amino acids with a calculated molecular weight of 66.3 kDa, which correlates very closely with the molecular mass estimated using a number of purified preparations of CK in yeast (Brostrom and Browning, 1973; Hosaka et al., 1990; Kim et al., 1998). Overexpression of the *CKI1* gene in yeast and *E. coli* caused increases in both CK and EK activity, further supporting the idea that the two activities reside on the same protein. On the other hand, inactivation of the *CKI1* gene caused yeast cells to lose all of their CK activity and most of their EK activity, indicating that there is only one gene responsible

for CK activity but that a second gene must exist to account for the remaining EK activity. The expression of *CKI1* has been shown to be transcriptionally repressed by *myo*-inositol and choline (Hosaka et al., 1990; McMaster and Bell, 1994b), and at exogenous choline concentrations above 100 μM the reaction becomes rate-limiting for PC synthesis (McMaster and Bell, 1994b).

In contrast to the yeast system, which contains only one gene for CK, there have been a number of cDNAs cloned from mammalian systems. Three cDNAs from rat tissues have been shown to encode CK activity. Rat CKI1 and CKI2 encode proteins of 435 amino acids and 453 amino acids, respectively, and are splice variants of the same gene that are expressed in the liver (Uchida, 1994; Uchida and Yamashita, 1992). A third cDNA, CKI3, which arises from a second gene, was cloned from rat kidney and encodes a protein of 394 amino acids (Aoyama et al., 1998b). To date, there has been only one human CK cDNA isolated, which was cloned from a glioblastoma cDNA library by complementation of the yeast *cki* mutation (Hosaka et al., 1992). The human enzyme encodes a protein of 456 amino acids with identity to the yeast enzyme in the carboxy terminus. There have been two cDNAs isolated from mouse embryo which demonstrate homology with the two rat genes (Aoyama et al., 1998a). All of the CK cDNAs cloned to date can catalyze the phosphorylation of both choline and ethanolamine, although in all cases the specificity is higher for choline. It still remains to be seen exactly how many isoforms actually exist in mammalian cells and what role each plays in the biosynthesis of PC and PE.

b. CTP:phosphocholine cytidylyltransferase (CT)

CTP:phosphocholine cytidylyltransferase (CT) catalyzes the key regulatory step in PC biosynthesis, namely the synthesis of CDP-choline from phosphocholine and CTP in a reaction which releases pyrophosphate (Figure 1). Because it is the rate-limiting step in the pathway, it has been extensively studied since its discovery in 1956 (Kennedy and Weiss, 1956). It was demonstrated early that CT exists in both membrane-bound and soluble forms (Wilgram and Kennedy, 1963) and its exact intracellular location has been the subject of numerous studies. It was also discovered that the enzyme was lipid activated (Fiscus and Schneider, 1966), and later studies showed that CT was rate-limiting (Vance et al., 1980) and regulatory (Sleight and Kent, 1980) for PC biosynthesis. Many early purification attempts failed due to the tendency of CT to aggregate and lose activity. However, purification of CT from rat liver cytosol was finally achieved when the enzyme was treated as if it were a membrane-bound enzyme by the addition of lipids and detergents (Feldman and Weinhold, 1987; Weinhold et al., 1986). The purified enzyme was estimated by SDS-PAGE to have a subunit M_r of approximately 45 kDa and cross-linking studies suggested that the enzyme exists as an elongated dimer (Cornell, 1989; Weinhold et al., 1989). In addition, purified CT was stimulated 10-fold in the presence of PC:oleic acid vesicles (Weinhold et al., 1986).

The first CT gene to be cloned was that from *S. cerevisiae*, *PCT1*, which was isolated from a genomic library by complementation of a mutant defective in CT activity (Tsukagoshi et al., 1987). The *PCT1* gene product encodes a protein of 424 amino acids with a predicted molecular weight of 49.4 kDa. Subsequently, a cDNA from rat liver was cloned by oligonucleotide-directed polymerase chain reaction (CTα) and was shown to contain a central region that is highly homologous to yeast CT (Kalmar et al., 1990). This

region has since been shown to be the catalytic domain and has been found in all cytidylyltransferases cloned to date, with a number of residues being absolutely conserved. Other mammalian CTαs that have been cloned include those from human (Kalmar et al., 1994), mouse (Rutherford et al., 1993), and Chinese hamster (Sweitzer and Kent, 1994). Recently, a second isoform of CT has been identified, CTβ1, which is encoded by a second gene (Lykidis et al., 1998). A third isoform of CT, CTβ2, was cloned recently and is a splice variant of CTβ1 described above (Lykidis et al., 1999). Prominent among the conserved residues of the catalytic domain is the sequence HXGH, which is the defining element of a nucleotidyltransferase superfamily (Bork et al., 1995; Jones et al., 1986). It has been proposed that the HXGH sequence is important in CTP binding and transition-state stabilization (Veitch and Cornell, 1996; Veitch et al., 1998). A second sequence element, RTEGISTT, appears to be a signature motif specific to the CT family (Park et al., 1997).

Aside from the catalytic domain, CTα has three other characterized functional regions, which include a nuclear localization signal, a membrane-binding domain, and a phosphorylation domain. Although early studies localized membrane-associated CT to the ER and Golgi, there has been increasing evidence demonstrating a nuclear localization for the enzyme. CT has been localized to the nucleus in several cell lines and in primary rat liver slices through indirect immunofluorescence (Wang et al., 1993b; Wang et al., 1993c; Watkins and Kent, 1992) and has been shown to translocate to the nuclear membrane upon activation. In agreement with a nuclear localization, a 21-residue nuclear localization signal was recently identified and characterized in the N-terminus of

CTα, without which CT would not localize to the nucleus but was nonetheless active and able to complement a CTα deficiency (Wang et al., 1995).

Immediately C-terminal to the catalytic domain is a membrane-binding domain containing several amphipathic α-helices, which has been shown to mediate lipid binding and activation. Translocation of the enzyme from the soluble to particulate fraction is often correlated to increases in PC biosynthesis and similar effects are seen with the addition of exogenous lipids to the soluble enzyme. Most evidence supports an *in vivo* model of an inactive pool of soluble CT, which is activated upon translocation to the membrane in response to appropriate physiological signals (Kent, 1997; Tronchere et al., 1994). A number of agents have been shown to cause translocation to membranes, including phospholipase C (Sleight and Kent, 1980), DAG (Kolesnick and Hemer, 1990), phorbol esters (Utal et al., 1991), and fatty acids (Hatch et al., 1992). A number of studies have indicated that the membrane-binding domain is responsible for lipid activation *in vitro* and membrane binding *in vivo*. When the helical domain is removed by proteolysis (Craig et al., 1994), truncated by mutagenesis (Cornell et al., 1995; Wang and Kent, 1995b; Yang et al., 1995), or blocked by antibody (Wieder et al., 1994), rat CT is unable to be activated by lipids. In addition, a truncated enzyme that cannot translocate to membranes is constitutively active, suggesting that the lipid-binding domain functions in an inhibitory manner in the absence of stimulating lipids (Wang and Kent, 1995b).

The fourth functional domain is the phosphorylation domain, which is located at the C-terminus of CT. Mutagenesis studies demonstrated that phosphorylation of CT occurs on sixteen serine residues at the extreme C-terminus (MacDonald and Kent, 1994) and that mutation of all sixteen residues resulted in CT which lacked phosphorylation, but

was otherwise identical to wild type CT (Wang and Kent, 1995a). Phosphorylation of CT interferes with the lipid stimulation of enzyme activity *in vitro* (Yang and Jackowski, 1995) and correlates with a reduction of PC biosynthesis *in vivo* (Watkins and Kent, 1990; Watkins and Kent, 1991). Although the physiological significance of CT phosphorylation has been speculated upon, the role has not been delineated, nor have the *in vivo* kinases and phosphatases been identified.

As mentioned above, there have been two other isoforms of CT cloned, CTβ1 and CTβ2. CTα and CTβ1 have nearly identical amino acid sequence in the both the catalytic domain and the membrane interaction domain, although CTβ1 is somewhat shorter. CTα is a protein of 367 amino acids, while CTβ1 is 330 amino acids. Similar to CTα, CTβ1 is dependent on interaction with phospholipids for catalytic activity, which would be expected from the high degree of similarity in the membrane-binding domain. However, CTβ1 does not contain a nuclear localization signal and, consistent with this, was found outside the nucleus by indirect immunofluorescence (Lykidis et al., 1998). In addition, CTβ1 lacks most of the C-terminal phosphorylation domain; therefore is most likely not regulated through phosphorylation. CTβ2 encodes a 369-amino acid protein that is identical to CTβ1 over amino acids 1-320. In contrast to CTβ1, CTβ2 has a C-terminal domain that resembles the phosphorylation domain of CTα, and has been shown to be heavily phosphorylated *in vivo*. Unlike CTα, which is more ubiquitously expressed, both CTβ isoforms had tissue-specific distributions. In addition, CTβ isoforms were associated with the ER, while CTα was found predominantly in the nucleus. Taken together, these studies suggest that CTβ1 and CTβ2 may play a role in the cell that is distinct from CTα, although a recent study has demonstrated that when CTα is knocked out in macrophages,

CTβ2 is subsequently induced (Zhang et al., 2000). This indicates that the activity of CTβ2 may be capable of compensating for the absence of CTα.

c. Cholinephosphotransferase (CPT)

Cholinephosphotransferase (CPT) catalyzes the final step in the *de novo* synthesis of PC, transferring phosphocholine from CDP-choline to diacylglycerol (DAG) and releasing CMP (Figure 1). There are a number of proposed CPT activities in mammalian cells, which are shown in Figure 2. Aside from synthesizing PC, CPT is also thought to catalyze the transfer of phosphocholine to alkylacylglycerol to form PAF precursor, to alkenylacylglycerol to form PC plasmalogen, and to alkylacetylglycerol to form PAF. The intracellular location of CPT defines the site of PC synthesis. Although CPT has been reported most often to be associated with the ER, depending on the subcellular fractionation method and enzyme assay used, there have been a number of studies localizing CPT to other organelles, including the Golgi (Jelsema and Morre, 1978; Vance and Vance, 1988), mitochondria (Schlame et al., 1989; Sikpi and Das, 1987), plasma membrane (Hargreaves and Clandinin, 1987), and nucleus (Hunt et al., 2001). In addition, CPT activity has been reported in mitochondria-associated membrane fractions (Vance, 1990) and a rapidly sedimenting ER (Shore and Tata, 1977).

Numerous attempts have been made to purify CPT, but to date it has not been purified to homogeneity from any source. The need to solubilize the integral membrane-bound protein from biological membranes prior to purification results in its separation from required phospholipid activators. In addition, many detergents cause irreversible inactivation of CPT upon solubilization of the membrane. Kanoh and Ohno were the first to describe the solubilization and partial purification of CPT from rat liver microsomes in

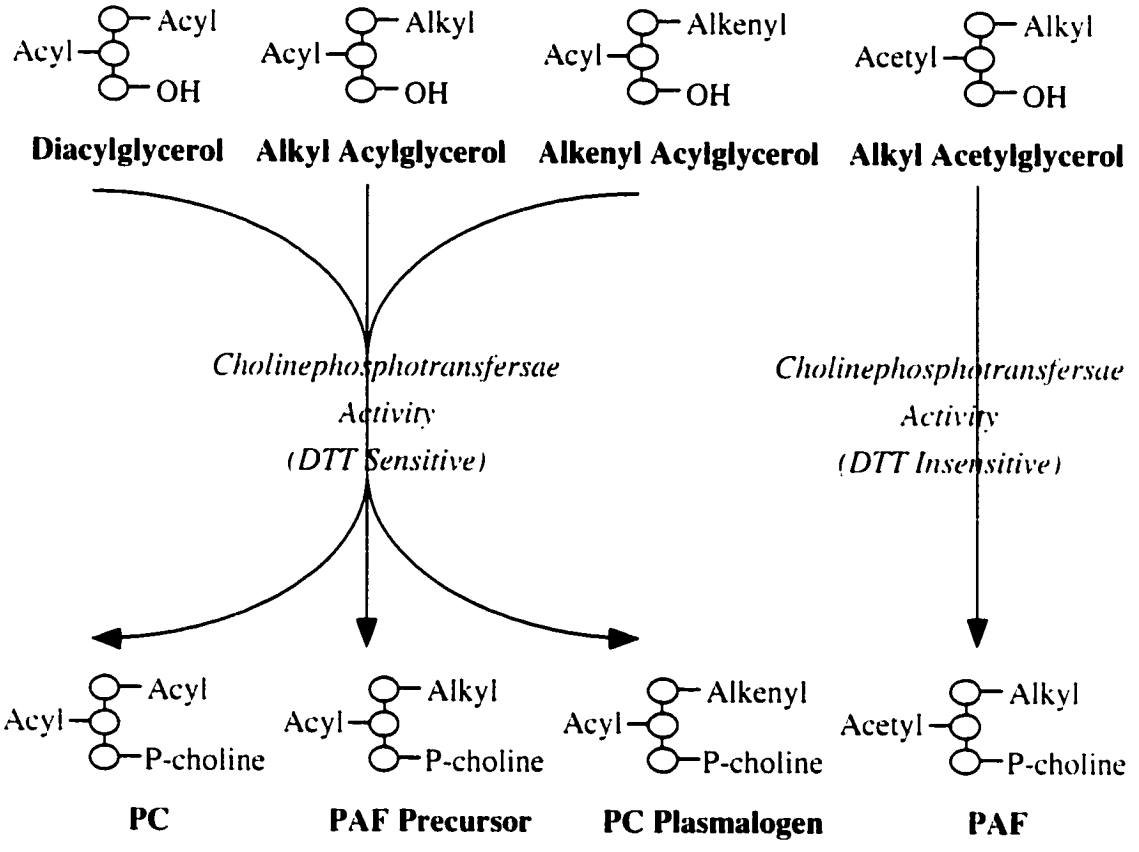


Figure 2. Proposed activities of mammalian CPT.

the presence of sodium deoxycholate (Kanoh and Ohno, 1976). The partially purified CPT required Mg^{2+} as a cofactor and microsomal phospholipids for maximal activity. In contrast, it has been shown that CPT from adult chicken brains is inactivated in the presence of sodium deoxycholate (Freysz et al., 1977). Another study demonstrated the solubilization of CPT from rat liver microsomes using octyl glucoside, but no further attempt was made to purify the solubilized enzyme (Radominska-Pyrek, 1978). Solubilization of CPT from hamster liver microsomes using Triton QS-15 allowed subsequent partial purification by chromatography (O and Choy, 1990). As seen previously, CPT had an absolute requirement for Mg^{2+} and interestingly, hamster heart CPT was unable to be solubilized by the same method. The most recent attempt utilized an ionic detergent (sodium deoxycholate) to solubilize CPT from rat liver microsomes, which was then partially purified using gel permeation chromatography (Ishidate et al., 1993). Both non-ionic (Triton X-100, octylglucoside) and zwitterionic (CHAPS) detergents irreversibly inactivated CPT at the solubilization step.

The first CPT gene to be cloned was that from *S. cerevisiae*, *CPT1*, *via* a colony autoradiography assay specific for CPT activity (Hjelmstad and Bell, 1987). The assay was based on the incorporation of radiolabelled CDP-choline into PC in permeabilized yeast colonies bound to filter papers, using endogenous DAG as the acceptor for phosphocholine. The radioactive products of the *in situ* reaction were characterized by extraction of the filter papers followed by TLC. Characterization of the assay demonstrated that endogenous DAG was not rate-limiting and that the delivery of radiolabelled CDP-choline to the enzyme was efficient (Hjelmstad and Bell, 1987). The assay was used to assess the CPT activity of yeast colonies mutagenized to 50% survival

with ethyl methanesulfonic acid, resulting in the identification of three complementation groups that were defective for CPT activity to varying degrees. The gene for *CPT1* was isolated by complementation of the *cpt1* defect by a plasmid (pRH1) contained in a yeast genomic library. Disruption of the *CPT1* gene caused a 5-fold reduction in CPT activity and showed that *CPT1* is nonessential for cell growth. The residual CPT activity exhibited a wild-type K_M for CDP-choline and was strongly inhibited by CMP. The mutants isolated that were defective in CPT activity possessed normal EPT activity, suggesting that different genes encode the two enzymatic activities. Subsequently, the *CPT1* gene was sequenced and found to contain an open reading frame encoding a protein of 407 amino acids with a predicted molecular weight of 46.3 kDa (Hjelmstad and Bell, 1990). The open reading frame was interrupted by a 92-base pair intron near the 5' end, which is rare in yeast. Yeast CPT was found to be composed of 50% hydrophobic residues, which is consistent with its exclusive localization to membrane subcellular fractions. Using a number of different structure prediction methods, CPT was hypothesized to contain seven transmembrane helices and an asymmetric distribution of hydrophilic regions with respect to the transverse plane of the membrane.

Analysis of Cpt1p by a Triton X-100 mixed micelle CPT assay revealed that CPT was specific in its use of CDP-choline as a substrate and was unable to utilize CDP-ethanolamine (Hjelmstad and Bell, 1991a). In addition, CPT exhibited an absolute requirement for divalent cations and a phospholipid cofactor. In the yeast strain used to isolate *CPT1*, the *EPT1* gene product (discussed in more detail in section B.1.c) was also shown to possess CPT activity. *In vitro*, the contribution to total CPT activity was shown to be approximately 50% for both Cpt1p and Ept1p. However, *in vivo* results

demonstrated that the *CPT1* gene product was predominantly a CPT, synthesizing 95% of total PC, and the *EPT1* gene product was predominantly an EPT, synthesizing only 5% of total PC (McMaster and Bell, 1994a; McMaster and Bell, 1994b). Construction and characterization of a series of *EPT1-CPT1* chimeric enzymes revealed internal linear segments of the enzymes that conferred substrate specificity and structural determinants of lipid activation (Hjelmstad et al., 1994). The active site was localized to the linear segment between residues 58 and 229 of Cpt1p, while the DAG binding site was predicted to contain the first three membrane-spanning helices. The CDP-aminoalcohol specificity domain was predicted to lie within the cytoplasmic loop spanning residues 79 to 186 of Cpt1p. Previous *in vitro* studies predict that the active site of mammalian CPT faces the cytoplasm (Coleman and Bell, 1978; Vance et al., 1977). In microsomal vesicles in which the cytoplasmic face was exterior, treatment with inhibitors dramatically reduced CPT and EPT activity, demonstrating that the active sites of both enzymes are located on the cytoplasmic face.

Aside from *CPT1* and *EPT1* from *S. cerevisiae*, the only other cDNA isolated at the onset of this work that could catalyze the CPT reaction was that from soybean (*Glycine max*). The soybean cDNA, aminoalcoholphosphotransferase (AAPT1), was cloned by complementation of a yeast mutant defective in CPT activity (Dewey et al., 1994) and encodes a protein 33% identical to Cpt1p. AAPT1p was shown to utilize CDP-choline, but its ability to use CDP-ethanolamine has not been tested, although the addition of either excess unlabelled CDP-choline or CDP-ethanolamine decreased the CPT activity of AAPT1p. This behavior is analogous to that of the *EPT1* gene product, suggesting the

ability of AAPT1p to use both CDP-choline and CDP-ethanolamine as substrates. The role of AAPT1 as a CPT or EPT *in vivo* has yet to be established.

Database searches for known proteins with identity to the CDP-aminoalcohol binding region of Cpt1p identified sequences within *S. cerevisiae* EPT (*EPT1*), PI synthase (*PIS1*), and PS synthase (*PSS1/CHO1*); prokaryotic phosphatidylglycerol phosphate synthase (*pgsA*) and PS synthase (*pss*); soybean AAPT1; and rat PI synthase. Each of these enzymes catalyzes the synthesis of a phospholipid by the displacement of CMP from a CDP-alcohol by a second alcohol to form a phosphodiester bond. Alignment of sequences within the above enzymes revealed an absolutely conserved motif, $DG(x)_2AR(x)_8G(x)_3D(x)_3D$ (Williams and McMaster, 1998). Scanning alanine mutagenesis of the conserved residues within this CDP-alcohol phosphotransferase motif of *S. cerevisiae* Cpt1p revealed that alanine mutants of Gly^{114}, Gly^{127}, Asp^{131}, and Asp^{135} were inactive. In addition, mutants of Ala^{117} and Arg^{118} displayed reduced enzyme activity, while mutants of Asp^{113} displayed wild-type activity (Williams and McMaster, 1998). These results predict a catalytic role for the phosphotransferase motif that uses a general base reaction. Most likely, catalysis occurs through Asp^{131} or Asp^{135} *via* a direct nucleophilic attack of the hydroxyl of DAG on the phosphoester bond of CDP-choline that does not proceed through an enzyme-bound intermediate.

As mentioned above and shown in Figure 2, platelet-activating factor (PAF) can be synthesized *de novo* in a reaction catalyzed by a cholinephosphotransferase. This enzymatic reaction was first described in 1981 (Renooij and Snyder, 1981) and involves the transfer of phosphocholine from CDP-choline to 1-alkyl-2-acetyl-*sn*-glycerol to form PAF. A number of studies have subsequently characterized this enzymatic reaction and

have found that the CPT that synthesizes PAF (PAF-CPT) has distinct properties from the CPT that synthesizes PC (PC-CPT), suggesting that the two enzymatic activities might represent two distinctly different proteins. At the onset of this work, neither activity had been cloned or purified from any mammalian source and all studies relating to the *de novo* synthesis of PAF have been performed using total microsomal protein. The most significant difference found between the two enzymatic activities has been that, unlike the PC-CPT, which is inhibited by DTT, the PAF-CPT is not inhibited by DTT but in fact is slightly stimulated by DTT (Renooij and Snyder, 1981; Woodard et al., 1987). The PAF-CPT activity has been found in a wide variety of tissues and topographical studies have revealed that it is located on the cytoplasmic surface of the ER (Woodard et al., 1987), which is similar to the PC-CPT (Coleman and Bell, 1978; Vance et al., 1977). Both activities require Mg^{2+} for activity and, notably, the detergent deoxycholate inhibits PAF-CPT but stimulates PC-CPT (Francescangeli and Goracci, 1989; Woodard et al., 1987). The determination of the number and specificity of isoforms of CPT will only be resolved with the cloning of the mammalian cDNAs.

2. Catabolism

Aside from its obvious structural role in biological membranes, PC is a reservoir for a number of important intracellular signalling molecules. Signalling molecules are released through the action of various phospholipases, whose products are shown in Figure 3. Phospholipase A_1 hydrolyzes the ester bond of phospholipids at the *sn*-1 position, releasing a fatty acid and a 2-lysophospholipid. Phospholipase A_2 catalyzes the analogous reaction at the *sn*-2 position of phospholipids, releasing a fatty acid and a 1-lysophospholipid. In yeast, both phospholipase A activities are present in the same

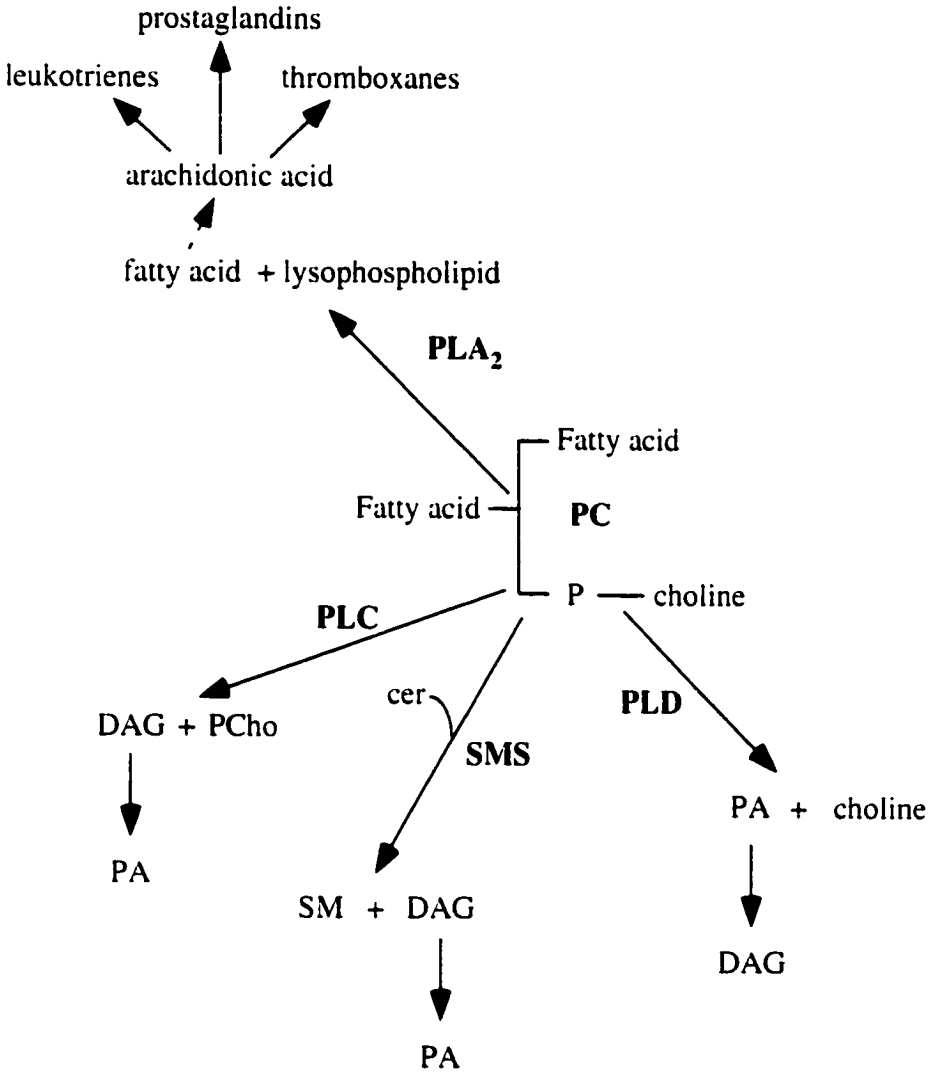


Figure 3. Catabolism of phosphatidylcholine. PC (pictured in center) is a reservoir for a number of signalling molecules. PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; SMS, sphingomyelin synthase; DAG, diacylglycerol; PCho, phosphocholine; SM, sphingomyelin; PA, phosphatidic acid; cer, ceramide.

enzyme, phospholipase B. Phospholipase C hydrolyzes the phosphodiester bond at the *sn*-3 position of phospholipids, releasing DAG and the corresponding phosphorylated head group. Similar products are produced *via* the action of sphingomyelin synthase, which transfers phosphocholine from PC to ceramide in the synthesis of sphingomyelin. Phospholipase D hydrolyzes phospholipids to phosphatidic acid and their free head groups. Finally, PC can be utilized as an acyl donor in the synthesis of cholesteryl ester and triglyceride in reactions catalyzed by lecithin:cholesterol acyltransferase (LCAT) and yeast Lro1p, respectively. These catabolic reactions in relation to PC are discussed in more detail in the following sections.

a) Phospholipase A_2

Phospholipase A_2 (PLA_2) describes not only one enzyme, but a large family of enzymes which vary in size, properties, and function. The unifying feature of all PLA_2s is their specificity for hydrolyzing the ester bond of phospholipids at the *sn*-2 position. The products of the reaction are free fatty acid and lysophospholipid, both of which have important roles in the cell. Free fatty acids such as oleic acid and arachidonic acid can be important as stores for energy in the cell. More specifically, arachidonic acid has been shown to be involved as a second messenger (Berk and Stump, 1999; Gijon and Leslie, 1999) and also serves as the precursor of eicosanoids, which are potent mediators of inflammation and signal transduction (Austin and Funk, 1999; Bingham and Austen, 1999; Devillier et al., 1999). The lysophospholipid produced in the PLA_2 reaction is important in cell signalling, phospholipid remodelling, and membrane perturbation (Balsinde et al., 1997; Moolenaar et al., 1997).

There are 11 groups of PLA_2s (I-XI), divided based on molecular weight, sequence similarity, calcium dependence, and cellular localization. The groups can be broadly separated into two categories based on catalytic mechanism. Groups I, II, III, V, IX, X, and XI all utilize a catalytic histidine for hydrolysis of the *sn*-2 ester bond. All members of these groups are secreted, low molecular weight (12-18 kDa) enzymes with a requirement for millimolar amounts of Ca^{2+}. In addition, all are highly disulfide bonded, possessing between 5 and 8 disulfide bonds. Hydrolysis occurs through the activation and orientation of a water molecule by hydrogen bonding to the active-site histidine. A conserved aspartate is located adjacent to the catalytic histidine, and serves as a ligand of Ca^{2+}, which stabilizes the negatively charged transition state of the PLA_2 reaction (Arni and Ward, 1996; Scott et al., 1990; White et al., 1990). The secreted PLA_2s ($sPLA_2$s) were the first to be characterized, dating as far back as the 1890s, when the enzymatic activity was found in cobra venom (Stephens and Myers, 1898). The PLA_2 groups I, II, V, and X are all related evolutionarily, as indicated by their high sequence and structural homology.

In addition to the numerous venom PLA_2s, there have been 10 distinct $sPLA_2$s isolated from mammalian sources. The first cloned mammalian $sPLA_2$ was from porcine pancreas and belongs to group IB (Seilhamer et al., 1986). Because of its high expression in the pancreas, the pancreatic $sPLA_2$ was thought to play a role only in dietary lipid digestion, but has since been shown to promote cell proliferation, cell migration, hormone release, and acute lung injury through the action of the M-type receptor (Hanasaki and Arita, 1999). Subsequently, a second $sPLA_2$ was cloned from human, mouse, rat, and guinea pig and belongs to group IIA (Murakami et al., 1997). Although

the group IIA $sPLA_2$ is detected at low levels in many cell types and tissues, it is found at very high levels in various inflamed tissues, tears and seminal plasma, as well as in the plasma and synovial fluids of patients with severe inflammatory diseases (Valentin and Lambeau, 2000). More recently, aside from its proposed role in inflammation, the group IIA $sPLA_2$ has been implicated in cell proliferation and apoptosis (Longo et al., 1999; Zhang et al., 1999). The group IIC $sPLA_2$ was cloned from rat and mouse testes by homology to group IIA (Chen et al., 1994a) and has been proposed to play a role in spermatogenesis (Chen et al., 1997). Database searches based on various $sPLA_2$ primary sequences revealed novel mammalian $sPLA_2$s, which have been labelled group IID (Ishizaki et al., 1999; Valentin et al., 1999b), IIE, and IIF (Valentin et al., 1999a). Group IID $sPLA_2$ expression appears to be regulated by inflammatory challenges and is expressed in several human and mouse tissues. The group V $sPLA_2$ has been cloned from human, rat, and mouse species (Chen et al., 1994b; Chen et al., 1994c) and is expressed at high levels in heart, lung, and placenta. It has also been found in macrophages and mastocytes, and has been shown to play a role in the release of lipid mediators of inflammation, such as arachidonic acid (Balboa et al., 1996; Han et al., 1999; Reddy et al., 1997). Another group with similar structural features, group X, was first cloned from humans (Cupillard et al., 1997) and has since been cloned from rat and mouse species as well (Valentin et al., 1999a). Compared with those from groups IB, IIA, and V, group X $sPLA_2$s have been shown to be potent at releasing arachidonic acid from resting cells with a concomitant increase in production of prostaglandin E_2 (Bezzine et al., 2000; Hanasaki et al., 1999). The group III PLA_2s are distantly related to those in groups I, II, V, and X and have many similar biochemical characteristics. Homologs have been

described in bee, lizard, jellyfish, and scorpion venoms and more recently, a human homolog has been cloned and characterized (Valentin et al., 2000). The human group III cDNA encodes a protein of 55 kDa, in contrast to the smaller (15-18 kDa), previously characterized protein from venom, and its biological role has not been studied in detail. The final two groups that use a catalytic histidine for hydrolysis are groups IX and XI, which were isolated from the marine snail *Conus magus* (McIntosh et al., 1995) and from plants (Stahl et al., 1999), respectively. There is limited sequence identity between these two groups and the ones described above aside from the conserved catalytic mechanism, and their associated biologies have not been determined.

The remaining groups of PLA_2s (groups IV, VI, VII, and VIII) utilize a catalytic serine for hydrolysis of the *sn*-2 ester bond. In contrast to the previously described secreted enzymes, the group IV PLA_2s are cytosolic ($cPLA_2$) and require only micromolar amounts of Ca^{2+} for enzymatic activity. The first $cPLA_2$ ($cPLA_2\alpha$) was identified and characterized in 1986 from human neutrophils (Alonso et al., 1986) and platelets (Kramer et al., 1986) and its cDNA was subsequently cloned and sequenced from both human and murine sources (Clark et al., 1991; Sharp et al., 1991). The mammalian cDNA encodes a protein of 749 amino acids with a molecular weight of 85 kDa. $cPLA_2\alpha$ has a marked specificity for cleaving arachidonic acid at the *sn*-2 position of phospholipids (Clark et al., 1991; Diez et al., 1992). Therefore, it has a crucial role in the biosynthesis of eicosanoids, a group of important bioactive lipid mediators, including prostaglandins, thromboxanes, and leukotrienes. Indeed, $cPLA_2\alpha$ knockout mice are incapable of generating leukotrienes, prostaglandins, or PAF (Bonventre et al., 1997; Uozumi et al., 1997). Activation of $cPLA_2\alpha$ requires physiological concentrations of Ca^{2+}

and phosphorylation of serine^{505}. An N-terminal calcium-binding domain (CaLB) homologous to the C2 domain of protein kinase C and phospholipase Cγ is required for activity and has been shown to mediate translocation of cPLA$_2$α to the endoplasmic reticulum and nuclear membranes, but not the plasma membrane. Phosphorylation of serine^{505} is mediated by mitogen-activated protein (MAP) kinase and must occur prior to Ca^{2+}-dependent translocation. Two homologs of cPLA$_2$α have recently been cloned and characterized. The cPLA$_2$β enzyme is 114 kDa and appears to be calcium dependent in the same manner as cPLA$_2$α (Pickard et al., 1999; Song et al., 1999). In addition to its PLA$_2$ activity, it has also been shown to have PLA$_1$ and lysoPLA$_2$ activity. cPLA$_2$γ is a protein of about 61 kDa with no Ca^{2+} dependence (Pickard et al., 1999; Underwood et al., 1998). Unlike cPLA$_2$α, cPLA$_2$γ does not have specificity for arachidonic acid but, similar to cPLA$_2$β, cPLA$_2$γ is able to hydrolyze both acyl chains from PC.

In addition to the Ca^{2+}-dependent PLA$_2$s described above, a Ca^{2+}-independent, cytosolic PLA$_2$ also exists (iPLA$_2$). The cDNA encoding iPLA$_2$ was cloned concurrently from Chinese hamster ovary cells, P338D$_1$ macrophages, and pancreatic islets (Balboa et al., 1997; Ma et al., 1997; Tang et al., 1997) and was shown to encode a protein of approximately 85 kDa. Evidence based on the inhibition of iPLA$_2$ by bromoenol lactone (BEL) and specific antisense oligonucleotide inhibition has suggested that iPLA$_2$ plays a key role in phospholipid acyl chain remodelling (Balsinde et al., 1997; Balsinde et al., 1995). In support of a remodelling role for iPLA$_2$, the enzyme seems to have no preference for particular fatty acids, including arachidonic acid.

The final two groups of PLA$_2$s, VII and VIII, are the PAF-acetyl hydrolases. PAF is a very potent mediator of inflammation whose effects can be nullified by hydrolysis of

the *sn*-2 acetyl group to form lyso-PAF. The PAF-acetyl hydrolases range in size from 26-45 kDa and both secreted and intracellular forms have been isolated. Similar to the group VI enzymes ($iPLA_2$), no members of groups VII or VIII require Ca^{2+} for activity.

b) Phospholipase C/ Sphingomyelin synthase

Phospholipase C (PLC) hydrolyzes the phosphodiester bond at the *sn*-3 position of PC, releasing DAG and phosphorylated choline (Figure 3). Similar products are produced *via* the action of sphingomyelin synthase (SMS), which transfers phosphocholine from PC to ceramide for the synthesis of sphingomyelin, releasing DAG. Neither activity has been cloned or purified from any eukaryotic source; therefore it has been a matter of debate as to which of the enzymes is responsible *in vivo* for this activity. The product of the reaction, DAG, has been shown to be an important intracellular signalling molecule. Many of the studies regarding the putative PC-PLC utilized the inhibitor, D609, which was previously believed to be specific for the enzymatic reaction catalyzed by PC-PLC. However, a recent study demonstrated that in addition to inhibiting PC-PLC activity, D609 could also inhibit SMS activity, and therefore many of the characteristics attributed to PC-PLC could in fact be characteristics of SMS (Luberto and Hannun, 1998). Resolution of the characteristics of PC-PLC and/or SMS awaits their cloning or purification from a eukaryotic source.

c) Phospholipase D

Phospholipase D (PLD) is a widely distributed enzyme that catalyzes the hydrolysis of PC to phosphatidic acid (PA) and choline. There is evidence that PA itself can act as a second messenger, as an activator of protein kinases (English et al., 1996), or as a direct regulator and activator of Raf-1 (Ghosh et al., 1996). PA has been shown to have roles in

secretion (Stutchfield and Cockcroft, 1993), DNA synthesis, and cell proliferation (Moolenaar et al., 1986; Nishizuka, 1995; Yu et al., 1988). PA can be dephosphorylated through the action of PA phosphohydrolase to form DAG, which is implicated in a number of cellular processes. For example, DAG derived from PC can regulate certain protein kinase C (PKC) isoforms (Nishizuka, 1995). PA can also be hydrolyzed by PLA_2 to form lysophosphatidic acid (LPA), which is a potent regulator of cell proliferation and is a potent mitogen (Guo et al., 1996).

Although yeast are known to contain multiple PLD activities, only one enzyme exists that is specific for hydrolyzing PC. The PC-specific activity (*SPO14*) was first identified in yeast extracts and was found to be regulated by glucose (Ella et al., 1995). *SPO14* was originally cloned and characterized as a gene required for meiosis and spore formation, in that *spo14* mutants were shown to be defective in the completion of meiotic divisions and subsequent gamete differentiation (Honigberg et al., 1992). Molecular analysis of the *SPO14* gene revealed that it encoded a PC-specific PLD. The *SPO14* gene encodes a protein of 1683 amino acids with a predicted molecular weight of 195 kDa (Waksman et al., 1996). No further PC-PLD activity was found in *spo14* mutants, suggesting that it is the only PC-PLD in *S. cerevisiae*. Similar to mammalian PLD1, Spo14p enzymatic activity is activated by PIP_2 (Rose et al., 1995), but, in contrast, is not activated by ARF (Rudge et al., 1998). Closely related sequences have also been found in other yeast such as *S. pombe* and *Candidia albicans*. Evidence suggests that Spo14p also plays a role in secretion, which is discussed further in section I.C.b.

The first eukaryotic cDNA for PC-specific PLD was isolated from castor bean based on peptide sequence derived from a 90 kDa purified PLD (Wang et al., 1993a; Wang et

al., 1994). This led to the cloning of the first cDNA encoding human PC-PLD (Hammond et al., 1995). Two mammalian PLD genes with approximately 50% identity (*PLD1* and *PLD2*) have been identified and their products have been shown to occur as several splice variants (Exton, 1998). Database searches have shown that only one PLD gene appears to exist in worms, flies, and yeast, and sequence comparisons suggest that the mammalian genes arose through duplication of an ancestral gene after divergence occurred from the lower eukaryotes (Redina and Frohman, 1998). PLD1 encodes a 120 kDa protein that occurs as two alternatively spliced isozymes (a and b), and has been localized to the perinuclear regions, which include the Golgi, ER, and late endosomes. The splice variant, PLD1b, has a 38-amino acid deletion, and no functional or regulatory differences were observed between the two human splice variants (Hammond et al., 1997). PLD2 encodes a 105 kDa protein, which has been cloned in mouse, rat, and human tissues (Colley et al., 1997; Kodaki and Yamashita, 1997; Lopez et al., 1998). A splice variant for human PLD2 has also been cloned, and lacks 11 amino acids in its C-terminus (Steed et al., 1998). Both PLD1 and PLD2 require PIP_2 for activity. Sequence alignments involving various PLDs have identified a number of regions with conserved homology. The most highly conserved domain in the superfamily is the $H(x)K(x)_4D$ motif, or PLD domain, which is present in two copies in all known PLD enzymes. Mutagenesis has shown that these amino acids are critical for catalysis *in vitro* and for PLD function *in vivo* (Sung et al., 1997). In addition to the PLD domain, a Px domain has been mapped in the N-terminal region of both PLD1 and PLD2, and evidence demonstrates that it might be implicated in protein-protein interactions (Ponting and Kerr, 1996).

PLD1 isozymes are regulated *in vitro* by conventional PKC isoforms (PKC-α, -β, and γ), but not by other PKC isoforms. Mutagenesis and binding studies have shown that PKC interacts with a domain in the N-terminus of PLD1 (Min and Exton, 1998; Park et al., 1998; Sung et al., 1999), and that the activation of PLD1 by PKC *in vitro* does not require phosphorylation. In contrast, *in vivo* studies have shown that phosphorylation of PLD1 by PKC does indeed play a role in its regulation (Exton, 1998). Hence, PKC may phosphorylate another protein that activates PLD or promotes its interaction with PKC. PLD1 is also regulated by Rho and ADP-ribosylation factor (ARF), which are two subfamilies of GTPases in the Ras superfamily (Exton, 2000). PLD isozymes are regulated differentially by long-chain fatty acids such as oleate in that PLD1 is strongly inhibited by oleate (Hammond et al., 1997), whereas a brain isoform is stimulated (Massenburg et al., 1994). In contrast to the complex regulation of PLD1, the PLD2 isozyme exhibits high basal activity and appears less subject to control. PLD2 shows no response to PKC isoforms or Rho subfamily proteins, and exhibits little or no stimulation by ARF (Colley et al., 1997; Lopez et al., 1998).

A variety of agonists have been demonstrated to stimulate PLD activity *in vivo*, such as growth factors, agonists that activate certain heterotrimeric G proteins, cytokines, and phorbol esters. In addition, PI-specific PLC generates DAG, which in turn activates the conventional PKC isoforms. Therefore, changes in PI-PLC activity correlate with alterations in PLD activity (Hess et al., 1998; Lee et al., 1994; Yeo et al., 1994).

d) Triglyceride and cholesteryl ester synthesis

PC has also been shown to be involved in the esterification of cholesterol through the action of lecithin:cholesterol acyltransferase (LCAT). The enzymatic mechanism of

LCAT involves both phospholipase and acyltransferase activities in transferring the *sn*-2 acyl group from PC to cholesterol. This conversion of cholesterol to long-chain cholesteryl esters occurs mainly on the surface of high-density lipoproteins (HDL) and promotes cholesterol movement from tissues into HDL. LCAT can also use PE as the acyl donor for the formation of cholesteryl esters (Pownall et al., 1985). Recently, it has been discovered that PC can also act as the acyl donor for the esterification of DAG in the synthesis of triglycerides (TG) in yeast (Oelkers et al., 2000). Previous to this discovery, the only method by which cells were thought to make TG was through the action of diacylglycerol *O*-acyltransferase (DGAT), which uses acyl-CoA as the donor to esterify DAG. Deletion of four genes with similarity to DGAT in *S. cerevisiae* did not considerably reduce TG synthesis, indicating that another mechanism existed. Database searches using the human LCAT sequence yielded one gene with significant similarity, *LRO1* (LCAT-related open reading frame). The *LRO1* gene product catalyzes the transfer of the *sn*-2 acyl group from PC to DAG and serves as the major method by which yeast synthesize TG (Oelkers et al., 2000). Although it has significant similarity to LCAT, Lro1 is not a functional LCAT homologue as it has no role in sterol esterification and does not use ergosterol as a substrate. DGAT-independent TG synthesis exists in mammalian systems as well, as shown by the observation that a mouse with no DGAT activity still retains significant TG synthesis (Smith et al., 2000). It remains to be seen whether a mammalian homologue of *LRO1* exists.

3. Biological roles

In addition to its structural role and its role as a reservoir of signalling molecules, PC can also be secreted and complexed with various proteins in the synthesis of

macromolecules such as lung surfactant, lipoproteins, and bile. Lung surfactant is a lipid- and protein-containing substance that is secreted by the lungs to prevent the collapse of the alveoli when air is expelled (Goerke, 1974). Respiratory distress syndrome is a common cause of neonatal mortality that occurs due to a problem in surfactant production and/or secretion. The major component of surfactant is dipalmitoyl-PC, a novel form of PC that is not found in cell membranes. Synthesis of surfactant lipids and proteins is developmentally regulated in fetal lung and can be accelerated by glucocorticoids and other hormones (Rooney et al., 1994). A number of studies have demonstrated that dipalmitoyl-PC can be synthesized *in vitro* from dipalmitoylglycerol and CDP-choline *via* the action of a cholinephosphotransferase (Ide and Weinhold, 1982; Miller and Weinhold, 1981). Previous studies, however, have suggested that dipalmitoyl-PC is formed by a remodelling mechanism, which combines the actions of a phospholipase A_2 and lysophosphatidylcholine acyltransferase (Kottgen and van Golde, 1976; Oldenborg and van Golde, 1976). The method by which dipalmitoyl-PC is synthesized for secretion as a component of surfactant remains to be elucidated.

Lipoproteins are macromolecular complexes of lipids and proteins that deliver hydrophobic, water-insoluble lipids, such as triacylglycerol and cholesterol, from the liver and intestine to other tissues in the body. There are a number of classes of lipoproteins, varying in their relative compositions of protein, phospholipids, cholesterol, cholesteryl esters, and triacylglycerols. The amount of phospholipid present in these complexes ranges from 9-24% of the total weight and the PC present on the surface of high-density lipoproteins serves as a substrate for LCAT, which was discussed previously.

B. Phosphatidylethanolamine (PE)

Phosphatidylethanolamine (PE) is the second most abundant phospholipid in eukaryotic cells, comprising about 25% of cellular phospholipid mass (Raetz, 1986), with a notable exception being *Drosophila*, in which it is the most abundant phospholipid (Ishidate, 1997). In most bacteria, PE is also the most abundant phospholipid, comprising 75% of the cellular phospholipid (Raetz, 1986). The pathway for the *de novo* biosynthesis of PE, like PC, was elucidated by Eugene Kennedy in 1956. However, PE has not been studied as extensively as PC and therefore much less is known regarding its synthesis and biological roles.

1. Structure and biosynthesis

PE is comprised of a glycerol backbone with fatty acids esterified at the *sn*-1 and *sn*-2 positions. The third position is occupied by a phosphate group to which an ethanolamine head group is attached (Figure 4). Similar to PC, PE can be synthesized *via* two routes. The CDP-ethanolamine or Kennedy pathway for *de novo* biosynthesis of PE is analogous to that of PC (Kennedy and Weiss, 1956), with free ethanolamine being phosphorylated by ethanolamine kinase (EK) as the initial step in the pathway. Phosphoethanolamine is then converted to CDP-ethanolamine in a reaction catalyzed by CTP:phosphoethanolamine cytidylyltransferase (ET). The final step in the pathway is catalyzed by ethanolaminephosphotransferase (EPT) and involves the transfer of phosphoethanolamine from CDP-ethanolamine to DAG, thereby synthesizing PE and releasing CMP. The enzymes involved in the *de novo* biosynthesis of PE are discussed in more detail in the following sections.

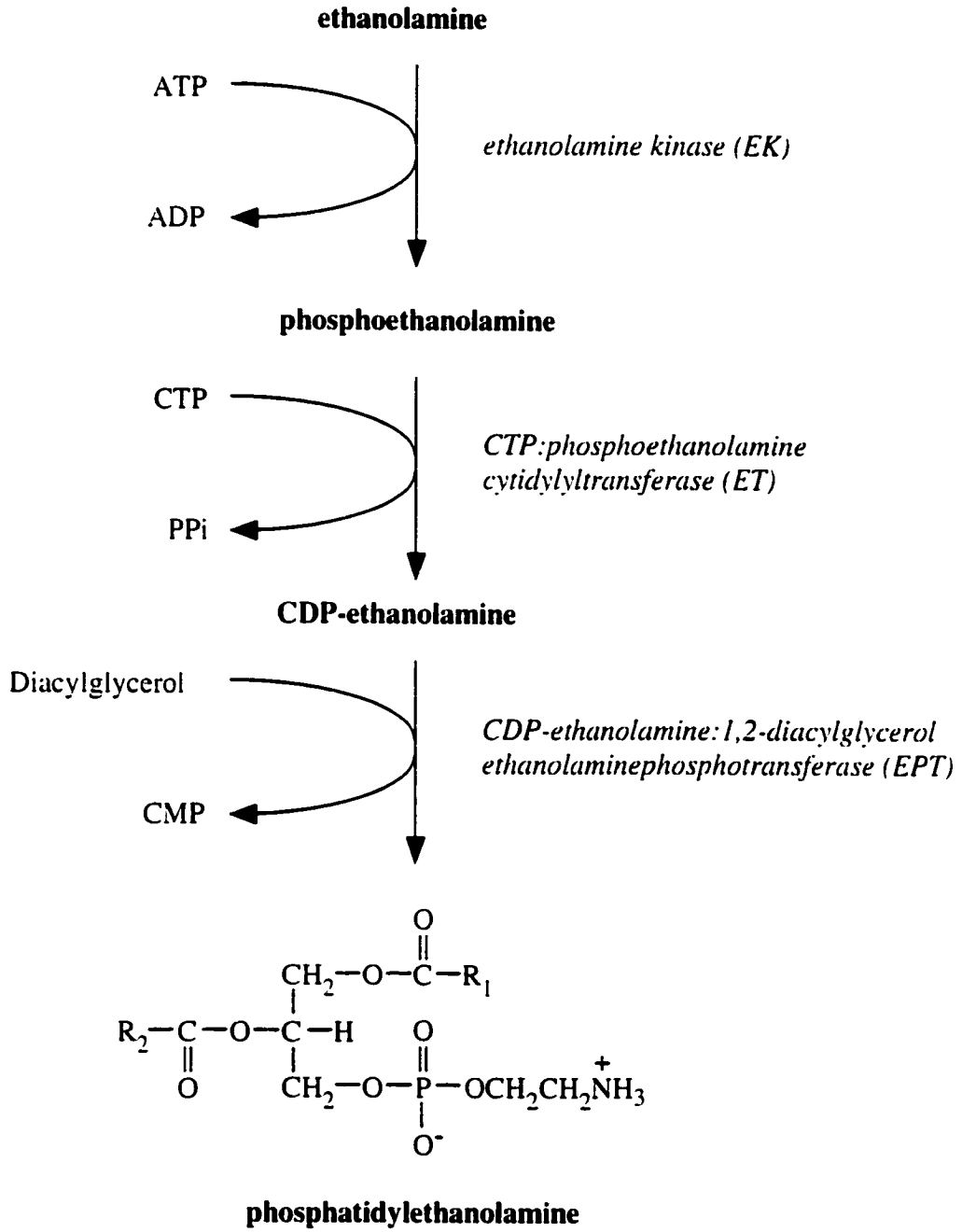


Figure 4. CDP-ethanolamine pathway for *de novo* PE biosynthesis. R_1 and R_2 denote long-chain fatty acids.

The second method by which cells can make PE is through the decarboxylation of phosphatidylserine (PS), a reaction catalyzed by phosphatidylserine decarboxylase (PSD). The enzymatic activity was first described in cell-free extracts of *Escherichia coli* (Kanfer and Kennedy, 1964). In most bacteria, the decarboxylation of PS is the only method by which PE is synthesized. Purified preparations of PSD from *E. coli* indicate that the enzyme consists of two subunits, an α subunit of 7332 Da and a β subunit of 28,579 Da (Dowhan et al., 1974) and the cloned bacterial PSD gene encodes a protein of 322 amino acids (Li and Dowhan, 1988). Contrary to the bacterial system, yeast cells contain two PSD genes. *PSD1* was cloned from *S. cerevisiae* by complementation of both yeast (Trotter et al., 1993) and *E. coli* (Clancey et al., 1993) mutants lacking PSD activity. The *PSD1* gene encodes a protein of 500 amino acids that is localized to the inner mitochondrial membrane (Zinser et al., 1991). *S. cerevisiae* strains with null alleles for *PSD1* exhibited no growth phenotype and only modest changes in lipid metabolism, suggesting that a second PSD gene must exist in yeast. The *PSD2* gene was identified using strains with *PSD1* null alleles that were mutagenized to produce new strains auxotrophic for ethanolamine. The *PSD2* gene was subsequently cloned and sequenced and was found to encode a protein of 1138 amino acids that was localized to the Golgi/vacuole membrane (Trotter et al., 1995; Trotter and Voelker, 1995). To date, only one mammalian PSD has been identified. The cDNA was cloned and found to encode a protein of 409 amino acids that, similar to yeast *PSD1*, is localized to the inner mitochondrial membrane (Kuge et al., 1996).

a. Ethanolamine kinase (EK)

Ethanolamine kinase (EC 2.7.1.82) catalyzes the analogous reaction to that of CK, phosphorylating ethanolamine as the initial step in the *de novo* synthesis of PE. As discussed above, all of the CK enzymes purified to date have significant EK activity in addition to CK activity (Ishidate et al., 1984; Porter and Kent, 1990; Uchida and Yamashita, 1990). These studies have supported the idea that both catalytic activities are found on the same protein. In contrast, there have been a number of reports suggesting the existence of a separate EK enzyme. Early kinetic studies on purified CK/EK preparations from rat liver indicated that although many properties were shared between the activities, CK and EK were separate enzymes (Brophy et al., 1977; Weinhold and Rethy, 1974). More recently, a partially purified preparation from human liver has been shown to possess 5-fold higher EK activity than CK activity (Draus et al., 1990). In addition, there has been a report of the purification of monomethylethanolamine kinase and dimethylethanolamine kinase from rat brain, although the significance of these enzymes is not known (Cao and Kanfer, 1991).

The gene encoding EK in *S. cerevisiae*, *EKI1*, was recently isolated and characterized based on its homology to *CKI1* (Kim et al., 1999). The *EKI1* gene product is 534 amino acids with 35% amino acid sequence identity to the yeast *CKI1*-encoded protein. Similar to CK, it is also predicted to be a cytosolic protein. In addition, yeast EK uses both ethanolamine and choline as substrates, although it phosphorylates ethanolamine at a rate 2-fold higher than that of choline. Deletion of the *EKI1* gene did not result in a growth phenotype and biochemical analyses of *ekiΔ ckiΔ* double mutants indicated that the *EKI1* and *CKI1* gene products encode all of the EK and CK activity in *S. cerevisiae* (Kim et al., 1999).

The situation in mammals appears to be different than that found in yeast. Although all of the CK enzymes isolated to date have significant EK activity, the data do not exclude the possibility of an ethanolamine-specific kinase in higher eukaryotes. In 1994, during the characterization of a *Drosophila* "bang-sensitive" paralytic mutant, *eas* (easily shocked), it was found to be lacking EK activity, while still retaining wild-type levels of CK activity (Pavlidis et al., 1994). This finding led to the cloning of the *Drosophila* cDNA for EK, which encodes a protein of 495 amino acids with significant similarity to CK/EK from yeast, human, and rat. Further characterization of the *eas* gene product as a fusion protein expressed in *E. coli* demonstrated it to be highly specific for phosphorylating ethanolamine (Uchida, 1997). The defect in EK activity is thought to alter membrane phospholipid composition, thereby causing the excitability defect.

Database searches using the *Drosophila* EK sequence revealed a human cDNA with significant similarity, which was termed *EKI1*. The *EKI1* cDNA encodes a protein of 452 amino acids and a predicted molecular weight of 49.7 kDa (Lykidis et al., 2001). Further searches using the *EKI1* cDNA revealed another open reading frame, *EKI2α*, which encodes a protein of 477 amino acids. There also exists a splice variant of *EKI2α*, *EKI2β*, which encodes a protein of 394 amino acids, but to date there are no data establishing either *EKI2α* or *EKI2β* as an ethanolamine-specific kinase. Overexpression of *EKI1* in COS-7 cells significantly increased EK activity, but did not increase CK activity, indicating that *EKI1* encodes a kinase that is highly specific for ethanolamine. In addition to the recently identified ethanolamine-specific kinases in *Drosophila* and human, there are mouse homologues to both *EKI1* and *EKI2*. These recent data have shown that in addition to the previously characterized CK enzymes (all of which also contain EK

activity), there exists at least one enzyme that is specific for phosphorylating ethanolamine.

b. CTP:phosphoethanolamine cytidylyltransferase (ET)

CTP:phosphoethanolamine cytidylyltransferase (ET) catalyzes the second step in the *de novo* biosynthesis of PE, which involves the condensation of CTP and phosphoethanolamine in a reaction that synthesizes CDP-ethanolamine and releases PP_i. Intracellular pyrophosphatases catalyze the hydrolysis of pyrophosphate, thereby driving the reaction in the direction of the synthesis of CDP-ethanolamine (Kennedy and Weiss, 1956). Although it has been less extensively studied than CT, it is thought to be the main regulatory step in the CDP-ethanolamine pathway. ET appears to be a largely cytosolic protein (Vermeulen et al., 1993), although it was shown by immunogold electron microscopy that ET is concentrated in areas that contain cisternae of the rough endoplasmic reticulum (van Hellemond et al., 1994). Unlike CT, ET has not been found in the nucleus. ET has been purified 1000-fold (Sundler, 1975) and to apparent homogeneity (Vermeulen et al., 1993) from post-microsomal fractions of rat liver. The molecular weight of the purified enzyme under denaturing conditions was estimated at 49.6 kDa, while gel filtration revealed the molecular weight of the native enzyme to be 100,000-120,000, suggesting that the enzyme occurs as a dimer (Vermeulen et al., 1993).

The gene encoding ET in *S. cerevisiae*, *ECT1*, was cloned by complementation of mutants that were unable to utilize extracellular ethanolamine for PE synthesis (Min-Seok et al., 1996). The *ECT1* gene product encodes a protein of 323 amino acids with a region containing significant similarities to the conserved catalytic domain of both yeast and rat CT. In contrast to CT, yeast ET does not appear to contain an amphipathic helical

domain, although, interestingly, there is preliminary evidence that, like rat liver ET, yeast ET may associate with the ER (Min-Seok et al., 1996). cDNAs encoding ET activity have also been cloned from mammalian sources. The human cDNA was cloned by complementation *in vivo* of a yeast mutant which was disrupted for *ECT1* (Nakashima et al., 1997). The cDNA was isolated from a human glioblastoma cDNA expression library and encodes a protein of 389 amino acids with 36% identity to the yeast ET. More recently, a cDNA was cloned by PCR amplification from rat liver with 89% identity to the human ET cDNA (Bladergroen et al., 1999). The 404-amino acid protein also showed significant similarity to other cytidylyltransferases, including rat CT and *Bacillus subtilis* glycerol-3-phosphate cytidylyltransferase.

c. Ethanolaminephosphotransferase (EPT)

Ethanolaminephosphotransferase (EPT) catalyzes the analogous reaction to CPT, transferring phosphoethanolamine from CDP-ethanolamine to DAG in the final step of the *de novo* synthesis of PE (Figure 4). As described above, the reaction catalyzed by ET, and not EPT, is generally accepted as the regulatory step in PE synthesis. However, under certain conditions in which DAG levels are decreased, PE synthesis is also decreased by a mechanism consistent with a rate-limiting role for EPT (Tijburg et al., 1989). The subcellular location of EPT is the site at which PE is synthesized. Although most studies localize EPT to the endoplasmic reticulum, its activity has also been found in the Golgi (Vance and Vance, 1988). As mentioned above for CPT, there have been numerous studies as to whether EPT and CPT activities are encoded by the same or separate proteins in eukaryotic cells. Isolation of a Chinese hamster ovary (CHO) mutant with low EPT activity but normal CPT activity gave the first genetic evidence that the

phosphotransferase reactions are catalyzed by two different enzymes (Polokoff et al., 1981). The mutant was also defective in the synthesis of ethanolamine plasmalogen, suggesting that the biosynthesis of PE and ethanolamine plasmalogen could occur *via* the same enzyme.

Numerous attempts have been made to purify EPT, but to date it has not been purified to homogeneity from any source. The first such attempt described the solubilization and partial characterization of both EPT and CPT from rat liver microsomes in the presence of sodium deoxycholate (Kanoh and Ohno, 1976). There were several significant differences in the properties of EPT and CPT, consistent with the two transferases being separate enzymes. Other early studies used octyl glucoside to solubilize EPT from rat liver microsomes, but no attempt was made to purify the solubilized enzyme (Radominska-Pyrek, 1978). Subsequently, buffered solutions containing octyl glucoside or Triton X-100 were used to solubilize and partially purify EPT from rat brain microsomes (Vecchini et al., 1987). Glycerol and DAG were found to stabilize the partially purified EPT, similar to that seen with partially purified CPT from sarcoplasmic reticulum (Cornell and MacLennan, 1985). Recently, there have been claims that EPT has been purified to electrophoretic homogeneity in a catalytically active form using high concentrations of non-ionic detergents (Mancini et al., 1999). The purified enzyme had a molecular weight of 38 kDa by SDS-PAGE and catalyzed both the EPT and CPT reactions, although its preference was for catalyzing the EPT reaction. However, the claim of a homogeneous preparation of EPT is only supported by SDS-PAGE, which is insensitive to small impurities; therefore further studies are necessary.

The only EPT to be cloned at the onset of this work was that from *S. cerevisiae*, *EPT1*, *via* a colony audioradiography assay developed for the cloning of *CPT1* (Hjelmstad and Bell, 1988). The assay was based on the incorporation of radiolabeled CDP-ethanolamine into lipid products in permeabilized yeast colonies bound to filter papers, using endogenous DAG as the acceptor for phosphoethanolamine. The assay was used to assess the EPT activity of colonies mutagenized with ethyl methanesulfonate, resulting in the identification of five complementation groups that were defective for EPT activity to varying degrees. The gene for *EPT1* was isolated by complementation of the *ept1* defect by a plasmid contained in a yeast genomic library. Subsequently, the *EPT1* gene was sequenced and found to contain an open reading frame for 391 amino acids with a predicted molecular weight of 44.5 kDa (Hjelmstad and Bell, 1991b). The *EPT1* gene product is 54% identical to *CPT1* and 32% identical to soybean *AAPT1*. Similar to *CPT1*, the open reading frame was interrupted by an intron near the 5' end, implying that the two genes may have risen from a duplication event. Structural predictions suggest a model very similar to the one predicted for *CPT1*, with seven membrane-spanning α-helices and the active site exclusively located on the cytoplasmic surface. Analysis of *CPT1*-*EPT1* chimeric enzymes mapped the CDP-aminoalcohol binding site of the *EPT1* gene product to the first soluble loop ranging from amino acids 70-172, while the DAG binding domain corresponds to the first three membrane-spanning helices (Hjelmstad et al., 1994; McMaster and Bell, 1994a).

Mixed micelle analysis of the *EPT1* gene product demonstrated that, *in vitro*, Ept1p is capable of utilizing not only CDP-ethanolamine, but also CDP-monomethylethanolamine, CDP-dimethylethanolamine, and CDP-choline as substrates

(Hjelmstad and Bell, 1991a). However, *in vivo* labelling studies utilizing *S. cerevisiae* cells defective in either the *EPT1* or *CPT1* gene demonstrated that Ept1p almost exclusively synthesized PE (McGee et al., 1994; McMaster and Bell, 1994a). The *EPT1* gene product contributed to only 5% of PC synthesis *in vivo*.

2. Catabolism

There is much less known about the hydrolysis of PE by phospholipases. The same phospholipases exert their effects on PE as those for PC (Figure 3). Various PLAs have been shown to have a greater affinity for cleavage of the *sn*-2 ester bond of PE than other phospholipids. In addition, there have been several reports demonstrating the activity of a PLC specific for PE hydrolysis (Kiss, 1992a; Kiss, 1992b).

In addition to the PC-specific PLDs that have been isolated from mammalian sources, there is growing evidence of a PLD specific for the hydrolysis of PE. In *S. cerevisiae* that are disrupted in the *SPO14/PLD1* gene (PC-PLD), a second PLD activity has been detected that has an absolute requirement for Ca^{2+} (Mayr et al., 1996; Waksman et al., 1997). This second PLD, named PLD2, preferentially hydrolyzes PE and PS and, unlike PLD1 from yeast, is unaffected by PIP_2 and does not catalyze a transphosphatidylation reaction. Subcellular distribution studies have localized yeast PLD2 to both the cytosol and particulate fractions (Mayr et al., 1996; Waksman et al., 1997). Although the cellular function of yeast PLD2 is unknown, it is believed that it represents the mitochondrial PLD activity first assayed in the 1970s, which implicated the enzyme in glucose repression of mitochondrial function (Dharmalingam and Jayaraman, 1971).

There is also evidence for PLD-mediated hydrolysis of PE in intact mammalian cells that is distinct from the PC-PLD activity of PLD1 and PLD2. In a number of cell types, several agents such as phorbol esters (Kiss and Anderson, 1989), sphingosine, ATP (Kiss and Anderson, 1990), and oxidants (Kiss and Anderson, 1994; Madesh et al., 1997) were shown to stimulate the production of PA and ethanolamine from PE under conditions that exclude PLC activity. Although relatively little is known about the regulation of PE-PLD, one study demonstrated that PKC-α has a major role in the mediation of the stimulatory phorbol ester effect on PE hydrolysis (Mukherjee et al., 1996). Subsequently, it was shown that phorbol ester can stimulate PE hydrolysis *via* a PKC-catalyzed phosphorylation mechanism or a phosphorylation-independent mechanism (Deli and Kiss, 2000). Together, the identification of yeast PLD2 as well as a number of mammalian studies suggests that PE-PLD may represent a new class of PLD enzymes that are structurally distinct from the PC-PLDs.

3. Biological roles

Unlike the situation for PC, there are few biological roles associated with PE. However, a recent study has demonstrated that PE present in the plasma membrane can act as a regulator of the dynamic movement of cytoskeletal proteins (Emoto et al., 1996). Generally in the plasma membrane, aminophospholipids such as PE are concentrated on the inner leaflet, while choline-containing phospholipids such as PC are found on the outer leaflet. However, a three-fold increase in PE on the outer leaflet of the plasma membrane is seen in dividing CHO cells upon entry into the late telophase/G1 phase (Kobayashi and Pagano, 1989). Using a peptide that specifically recognizes PE, it was

demonstrated that PE was exposed on the cell surface specifically at the cleavage furrow during the late telophase of cytokinesis (Emoto et al., 1996).

C. Phosphatidylcholine and vesicular trafficking

SEC14 is an essential gene that codes for the major PC/phosphatidylinositol (PI) transfer protein in yeast (Aitken et al., 1990; Bankaitis et al., 1990). Sec14p is a cytosolic factor of approximately 35 kDa that stimulates essential Golgi secretory functions (Bankaitis et al., 1989) and is required for secretory protein transport from a late Golgi compartment (Franzusoff and Schekman, 1989). Ablation of *SEC14* function prevents Golgi-mediated protein transport and results in cell death (Cleves et al., 1991; Kearns et al., 1998; Phillips et al., 1999). Due to the essential nature of *SEC14*, studies of Sec14p have been facilitated by the isolation of a temperature-sensitive allele of *SEC14*, *sec14-1*^{ts}, in which Gly^{266} of Sec14p is replaced by Asp. Expression of the *sec14-1*^{ts} allele renders both the PI and PC transfer activities of Sec14p labile *in vitro* (Bankaitis et al., 1990; Cleves et al., 1989). Yeast strains containing the *sec14-1*^{ts} allele are unable to grow at 37°C and have a defective secretory pathway, as shown by their inability to secrete invertase. The use of the temperature-sensitive allele of *SEC14*, *sec14-1*^{ts}, allowed a search for mutations in other genes that would allow survival in the face of a non-functional *SEC14* gene product. This screen resulted in the isolation of several *sec14*^{ts} bypass suppressor genes (Cleves et al., 1991). The first such gene isolated was *SAC1*, a gene which also interacts with yeast actin defects (Cleves et al., 1989). Three of the bypass suppressor genes were found to code for the Kennedy pathway enzymes: choline kinase (*CKI1*), CTP:phosphocholine cytidylyltransferase (*PCT1*), and cholinephosphotransferase (*CPT1*). The observation that mutations in the CDP-choline

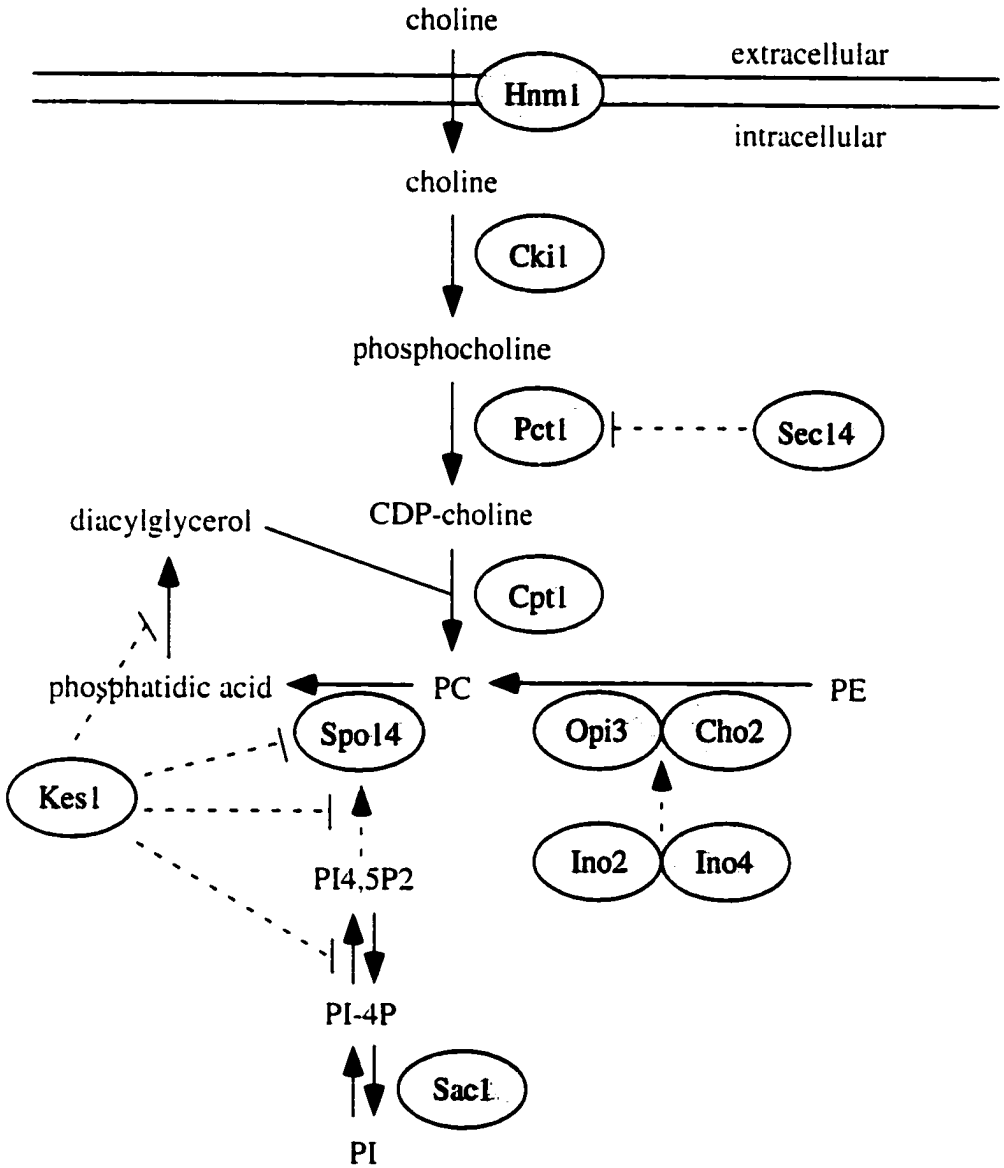


Figure 5. Role of phosphatidylcholine in the regulation of Golgi-derived vesicular transport. Metabolic steps are indicated by arrows. Known yeast proteins whose activities have been demonstrated *in vitro* and/or *in vivo* are indicated in the ovals. Predicted inhibitory events are illustrated by dashed lines and activation events by dashed arrows.

pathway bypass the Sec14p requirement provided the first evidence linking phospholipid biosynthesis and intracellular phospholipid transport to secretory pathway function (Figure 5). Interestingly, none of the enzymes of the Kennedy pathway enzymes for the synthesis of PE were isolated during the screen, and intentional disruption of the yeast *EKI1* or *EPT1* gene products did not rescue cells from the requirement for a functional *SEC14* (Cleves et al., 1991; Kim et al., 1999). In addition, mutations in the methylation pathway for PC biosynthesis did not bypass the requirement for Sec14p. Hence, the cellular requirement for *SEC14* could only be bypassed by inactivating genes in the Kennedy pathway for the synthesis of PC.

The ability of the *SEC14* bypass mutants to allow cell survival in the absence of a functional *SEC14* gene product is currently postulated to be due to alterations in Golgi DAG pool sizes, with increased DAG allowing growth in the absence of *SEC14*. However, both the *CPT1*- and *EPT1*-derived gene products directly consume DAG, yet inactivation of *CPT1* bypasses the cellular requirement for *SEC14* while inactivation of *EPT1* does not. This observation appears to be paradoxical, given the DAG pool size hypothesis. In support of the hypothesis that increased DAG concentration allows bypass of the *SEC14* requirement, defects in Sac1p have been shown to alter phospholipid metabolism so as to expand the pool of DAG in the Golgi (Kearns et al., 1997). In addition, expression of DAG kinase in strains carrying the *sec14-1*^{ts} allele further impaired secretory function.

Another of the genes whose inactivation bypasses the requirement for Sec14p was identified as *KES1*, which encodes Kes1p, a yeast member of the oxysterol binding protein family (Fang et al., 1996). Kes1p was demonstrated to act as a potent negative

effector of the Sec14p pathway for Golgi-derived transport vesicle biogenesis in yeast and was also identified as the agent through which the toxicity of CDP-choline pathway-derived PC to Golgi secretory function is mediated. To sum the data accumulated to date, it is hypothesized that DAG acts as a positive regulator of Sec14p-mediated vesicular transport, while PC acts oppositely as a negative regulator of this process.

2. PC-PLD in *S. cerevisiae*

SPO14 encodes the sole PLD in *S. cerevisiae* and is described in detail in section I.A.2.c. Based on earlier evidence that PLD plays an essential role in promoting Golgi-derived vesicle budding (Liscovitch and Cantley, 1995), recent studies have demonstrated that PLD activity is essential for yeast Golgi secretory function and that PLD involvement is essential in the Sec14p-bypass mutants. Although necessary, PLD catalytic activity is not sufficient for bypass of the Sec14p requirement. One interpretation of the PLD data is that PA, and not DAG, is the key lipid effector for Sec14p-dependent Golgi secretory function. In this hypothesis, the proposed DAG requirement is explained by DAG serving as a precursor to PA through the action of a DAG kinase (Kearns et al., 1997; Waksman et al., 1997). However, expression of *E. coli* DAG kinase in yeast specifically exacerbates *sec14* phenotypes and abolishes Sec14p-bypass phenotypes (Kearns et al., 1997). In addition, there is no evidence as yet to indicate that yeast can convert DAG to PA. On the other hand, it is possible that PLD-generated PA serves as a precursor to DAG production through the action of a PA phosphohydrolase (PAP). Although two pyrophosphatases have been described that exhibit PAP activity *in vitro* (Toke et al., 1998), the double-null strains do not exhibit Sec14p-bypass phenotypes. However, biochemical data indicate that yeast contain

additional, uncharacterized PAP activities. The elucidation of whether DAG or PA (or both) are required for Sec14p-dependent Golgi functions awaits the cloning and/or purification of DAG kinase and PAP from yeast.

II. Objective

Until recently, the enzymes involved in lipid synthesis and turnover have been primarily studied through subcellular fractionation and partial purification. The integral membrane-bound nature of many of these enzymes have made purification difficult because the detergents required to solubilize the proteins from the membrane bilayer often inactivate the enzyme. These studies have more recently become facilitated by ability to clone the cDNAs encoding the enzymes, which can subsequently be expressed in a number of systems, including bacterial, yeast, or mammalian systems. With the sequencing of a number of genomes, there has been a dramatic increase in the molecular tools available to study the enzymes involved in lipid synthesis and turnover. The objective of this study was to clone, express, and characterize the first mammalian cDNA(s) encoding a cholinephosphotransferase activity through comparison with the previously cloned *S. cerevisiae* choline- and ethanolaminephosphotransferases.

III. Materials and Methods

A. Materials

All molecular biology reagents used in this study were purchased from either New England Biolabs or Life Technologies. All materials used in the preparation of bacterial and yeast media were purchased from Difco Laboratories. Multiple Tissue Northern Blots™ I and II, ExpressHyb, and the λGT11 Quick-Clone cDNA libraries were obtained from Clontech. The GENETRAPPER® Positive Selection System and human brain cDNA library were products of Life Technologies. Immobilon™-P PVDF was purchased from Millipore. [Methyl-^{14}C]cytidine 5'-diphosphocholine and [ethanolamine 1,2-^{14}C]cytidine 5'-diphosphoethanolamine were purchased from American Radiolabeled Chemicals. [Methyl-^{14}C]-choline chloride, [α-^{32}P]dCTP (3000 Ci/mmol), and inorganic phosphorus 32 were purchased from NEN Life Science Products. [Ethanolamine 1,2-^{14}C]-ethanolamine hydrochloride was purchased from ICN. Lipids were purchased from Avanti Polar Lipids (Alabaster, AL), with the exception of di8:0 phosphatidic acid, which was purchased from Sigma Chemical Co.

1. Plasmids

A number of vectors were used to synthesize the plasmids used in this study. A list of the vectors, their usage, and the cell type in which they were used is shown in Table 1. The original EST containing the cDNA for hCEPT1 in a pBluescript vector was obtained from the American Type Culture Collection (Genbank™ accession number AA312638) and propagated in *E. coli* strain DH5α. The clone (2051 base pairs) was sequenced in its entirety on both strands and an open reading frame of 1248 base pairs was identified with similarity to yeast Cpt1p and Ept1p. The proposed coding sequence within the EST was

Table 1. Vectors used in this study.

Vector name	Usage	Cell types
pBluescript	Sequencing, subcloning	DH5α
pCR®2.1-TOPO®	Subcloning PCR products	TOP10
pET23a	Protein expression, subcloning	DH5α, BL21(DE3) pLysS
p416 GPD	High-copy yeast expression	DH5α, HJ091
pCDNA3	Mammalian expression, *in vitro* transcription/translation	DH5α, CHO-K1
pEGFP-N1	GFP fusion protein synthesis, mammalian expression	DH5α, CHO-K1
YFP-Golgi	Golgi localization	DH5α, CHO-K1
pCMV-SPORT	Human brain cDNA library	DH5α

amplified by PCR using the oligonucleotide primers 5'-GCGGGATCCATGAGTGGGCATCGATCAACA (forward) and 5'-GCGGTCGACTTAATGATTAGAATGAGCTGT (reverse), which have *Bam*HI and *Sal*I restriction sites, respectively, built in. The PCR product was subcloned into the pCR™2.1-TOPO™ vector (pAH4) as described in section B.1.e. The hCEPT1 open reading frame was excised from pAH4 with *Bam*HI and *Sal*I and subcloned into the *Bam*HI and *Sal*I sites of the *E. coli* expression vector pET23a (pAH5), resulting in the addition of an 11-residue, T7-epitope tag to the N-terminus of the protein. The T7-tagged version of hCEPT1 was excised from pAH5 using *Bgl*II and *Sal*I and subcloned into the *Bam*HI and *Sal*I sites of the constitutive yeast expression vector p416 GPD, creating pAH9. To create the mammalian expression vector pMM6, the T7-tagged version of hCEPT1 was excised from pAH5 using *Bgl*II and *Not*I restriction enzymes and subcloned into the *Bam*HI and *Not*I sites of pCDNA3, which drives expression from the CMV promoter. For subcellular localization studies, the hCEPT1 open reading frame, together with the N-terminal T7 tag, was amplified by PCR from pAH9 using the oligonucleotide primers 5'-GCAAGATCTATGGCTAGCATGACTGGTGGA (forward) and 5'-GCGCAGAATTCGATGATGATTAGAATGAGC (reverse), which have *Bgl*II and *Eco*RI restriction sites, respectively, built in. The PCR product was subcloned by TA cloning into the pCR™2.1-TOPO™ vector to create pAH19. The T7-tagged hCEPT1 open reading frame was excised from pAH19 using the restriction enzymes *Bgl*II and *Eco*RI and subcloned into the *Bgl*II and *Eco*RI sites of the mammalian expression vector pEGFP-N1, creating pAH20. Expression of pAH20 in mammalian cells results in the

production of a hCEPT1 protein with an N-terminal T7-epitope tag and a C-terminal green fluorescent protein.

The other set of plasmids created contained the various hCPT1 cDNAs. A human brain cDNA library that was directionally cloned into the CMV-SPORT eukaryotic expression vector was screened using the GENETRAPPER® kit as described in section III.4.a. Of the clones screened, there were five apparent full-length clones, which were sequenced in their entirety on both strands. Three of the clones contained an open reading frame of 1218 base pairs with identity to hCEPT1 and was designated hCPT1. Two of the clones contained the identical hCPT1 sequence with the exception of an insert of 27 base pairs at position 631 of the open reading frame. This insert causes a premature termination codon resulting in an open reading frame of 654 base pairs, which was designated hCPT1β. The open reading frames of all five GENETRAPPER® clones were amplified by PCR using the oligonucleotide primers 5'-GCCAGATCTATGGCGGCGGCGCCGGGGCC (forward) and 5'-GCCGTCGACTCAATCCATGTTATTCTGATG (reverse), which have *Bgl*II and *Sal*I restriction sites, respectively, built in. The PCR products were subcloned by TA cloning into the pCR®2.1-TOPO® vector, creating pAH10. The hCPT1 open reading frame was excised from pAH10 with *Bgl*II and *Sal*I and subcloned into the *Bam*HI and *Sal*I sites of pAH9, thereby replacing the hCEPT1 cDNA with the hCPT1 cDNA and placing a T7-epitope tag at the N-terminus of hCPT1 for constitutive expression in yeast (pAH12). The *Bgl*II/*Sal*I fragment excised from pAH10 was also subcloned into the *Bam*HI and *Sal*I sites of the pET23a vector, resulting in the addition of a T7-epitope tag to the N-terminus of hCPT1 (pAH21). To create the mammalian expression vector pAH23, the T7-tagged

version of hCPT1 was excised from pAH21 using *Bgl*II and *Not*I and subcloned into the *Bam*HI and *Not*I restriction sites of pCDNA3. Finally, to create the vectors for expression of hCPT1β, the hCPT1β cDNA was excised from pAH10 using *Bgl*II and *Sal*I and subcloned into the *Bam*HI and *Sal*I sites of pET23a (pAH27). The T7-tagged version of hCPT1 was excised from pAH27 with *Bgl*II and *Not*I and subcloned into the *Bam*HI and *Not*I sites of pCDNA3, creating pAH28. All PCR-derived products were also sequenced in their entirety.

2. Yeast Strains

Yeast strains used in this work are listed in Table 2. All CTY strains were the kind gifts of Dr. Vytas Bankaitis (University of North Carolina at Chapel Hill, Chapel Hill, USA). All YPP strains were the kind gift of Dr. Gerald Johnston (Dalhousie University, Halifax, Canada).

3. Antibodies

A number of antibodies were used in this study. The T7 mouse monoclonal (mT7) and T7 mouse monoclonal conjugated to horseradish peroxidase (T7-HRP) are directed towards an 11-amino acid peptide fused to the N-terminus of a number of proteins used in this study (Novagen). The peptide is the natural amino-terminal end of the T7 major caspid protein. To identify the endoplasmic reticulum, an anti-calnexin antibody was used, which is a rabbit polyclonal antibody raised against a 19-residue synthetic peptide based on the carboxyl terminus of canine calnexin (StressGen Biotechnologies Corp.). To identify the Golgi apparatus, FITC-labelled lentil (LcH) lectin was used, which was purchased from Sigma. LcH lectin is purified agglutinin from *Lens culinaris* that has an affinity for terminal α-D-mannosyl and α-D-glucosyl residues, which are concentrated in

Table 2. Yeast strains used in this study.

Yeast Strain	Genotype	Reference or Source
DBY747	**a** *his3-Δ1 leu2-3, 112 ura3-52 trp1-289*	Yeast Genetics Stock Center
HJ091	**a** *his3-Δ1 leu2-3, 112 ura3-52 trp1-289 cpt1::LEU2 ept1-1*	(Hjelmstad et al., 1994)
CMY133	**a** *ura3 leu2 his3 trp1 cpt1::LEU2 ept1::URA3* GAL^{-}	McMaster Lab
CTY1-1A	**a** *ura3-52 Δhis3-200 lys2-801 sec14^{ts}*	(Cleves et al., 1991)
CTY434	**a** *ura3-52 ade2-101 leu2-3,112 his4-519 sec14^{ts} cpt1::LEU2*	Bankaitis Lab
CTY468	**a** *ura3-52 his3-200 lys2-801 sec14^{ts} Δcct1::URA3*	Bankaitis Lab
CTY160	**a** *ura3-52 his3-200 lys2-801 sec14^{ts} ckl1*	Bankaitis Lab
CTY182	**a** *ura3-52 Δhis3-200 lys2-801 CPT1 EPT1*	(Cleves et al., 1991)
YPP649.7	**a** *ura3 sec7^{ts}*	Johnston Lab
YPP649.13	**a** *ura3 sec13^{ts}*	Johnston Lab
YPP649.15	**a** *ura3 sec15^{ts}*	Johnston Lab

the Golgi apparatus. The αC-CT antibody is a rabbit polyclonal antibody directed against the 45 amino acids at the C-terminus of rat CTα and was a gift from Dr. Martin Post at The Hospital for Sick Children in Toronto, Canada. Goat anti-mouse Texas Red (GAM-TxRed), goat anti-rabbit Texas Red (GAR-TxRed), goat anti-mouse FITC (GAM-FITC), and goat anti-rabbit FITC (GAR-FITC) secondary antibodies were products of Molecular Probes.

B. Methods

1. Bacterial protocols and recombinant DNA techniques

a. Preparation of chemically competent bacteria

Bacteria were grown overnight in 5 ml of Luria Bertani (LB) broth. The overnight culture was used to inoculate 500 ml LB broth and was grown at 37°C to an absorbance at 600 nm of 0.6. The bacteria were pelleted at 6000 x *g* for 15 minutes at 4°C and resuspended in one-third of the large culture volume of freshly prepared, sterile 0.1 M calcium chloride. After incubation on ice for 4 hours (swirling occasionally), the bacteria were pelleted and resuspended in one-thirtieth of the large culture volume of freshly prepared, sterile 0.1 M calcium chloride/ 15% glycerol. The bacteria were aliquoted after leaving on ice overnight at 4°C. Transformation efficiency of the bacteria was generally 1-5 x 10^6 colony forming units/μg plasmid DNA.

b) Transformation of chemically competent bacteria

Plasmid DNA was introduced into bacteria through the calcium chloride transformation method (Sambrook et al., 1989). An appropriate amount of DNA (20-50 ng ligation reaction or plasmid DNA) was added to 50-100 μl competent bacteria. The mixture was placed on ice for 30 minutes, heat-shocked at 42°C for 1 minute, and placed

on ice for 2 minutes. One milliliter of LB was added and the bacteria were allowed to recover for 30-60 minutes at 37°C. The bacteria were subsequently spread onto LB solid medium containing the appropriate antibiotic for plasmid selection and incubated overnight at 37°C.

c) Isolation of plasmid DNA from bacteria

Bacteria containing the desired plasmid were grown overnight in 2 ml LB broth containing the appropriate antibiotics. The culture was pelleted at 12,000 x *g* for 1 minute and resuspended in 100 µl of ice-cold 50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA. Next, 200 µl of fresh 0.2 M sodium hydroxide, 1% SDS was added, mixed by inversion, and placed on ice for 3 minutes. Ice-cold 3 M sodium acetate (150 µl, pH 5.2) was added, again mixed by inversion, and placed on ice for 5 minutes. The mixture was centrifuged at 12,000 x *g* for 5 minutes and the clear supernatant was transferred to a fresh tube. Then, 400 µl of phenol:$CHCl_3$:isoamyl alcohol (25:24:1) was added, the mixture was vortexed vigorously and centrifuged at 12,000 x *g* for 5 minutes. The top (aqueous) phase was transferred to a fresh tube and plasmid DNA was precipitated by adding 800 µl of ice-cold ethanol and centrifuging at 12,000 x *g* for 20 minutes at 4°C. The pellet was washed with 400 µl of ice-cold 70% ethanol, dried under a vacuum and resuspended in 30 µl sterile, deionized water (dH_2O) containing RNase A (20 µg/ml). This solution was incubated at 37°C for 15 minutes. Plasmid DNA was further purified for site-directed mutagenesis through the addition of 10 µl of 30% PEG-8000, 1.5 M sodium chloride. The mixture was placed on ice for 60 minutes, then centrifuged at 12,000 x *g* for 10 minutes at 4°C. The resulting pellet was successively washed with 70% ethanol, then 100% ethanol, dried under a vacuum and resuspended in 20 µl TE buffer.

d) Purification of DNA using GeneClean

DNA fragments and vectors for cloning were purified using the GeneClean II kit from Bio 101. The target DNA was excised from an ethidium bromide-stained agarose gel using a razor blade, placed in a microcentrifuge tube, and the approximate mass of the gel slice was determined. Three volumes of 5 M NaI solution were added to the gel slice and incubated at 55°C for 5 minutes to dissolve the agarose. Glassmilk (silica matrix) was vortexed to resuspend and 5 µl was added to the DNA solution. The suspension was incubated at room temperature for 10 minutes, with vortexing every 1-2 minutes to ensure the silica matrix stayed in suspension. The Glassmilk was pelleted by spinning at 12,000 x *g* for 5 seconds and washed 3 times with 700 µl NEW buffer. The washed pellet was resuspended in 5-10 µl of dH_2O and incubated at 55°C for 3 minutes to elute DNA. The suspension was centrifuged for 30 seconds and the eluate containing the purified DNA was transferred to a new microcentrifuge tube.

e) TOPO-TA Cloning

PCR products were ligated into the pCR®2.1-TOPO® vector using the TOPO-TA Cloning® kit purchased from Invitrogen Corporation. The plasmid vector (pCR®2.1-TOPO®) is supplied linearized with single 3'-thymidine (T) overhangs for TA cloning and topoisomerase I covalently bound to the vector for one-step cloning. PCR products were synthesized using *Taq* polymerase (Life Technologies) and GeneCleaned as outlined above. For those PCR products synthesized using Advantaq polymerase (Clontech), the GeneCleaned products were incubated at 72°C for 20 minutes in 1X PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 0.2 mM dNTP mixture, 1.5 mM $MgCl_2$, and *Taq* polymerase to add adenine (A) overhangs onto the PCR product. To perform the

ligation, 4 µl of PCR product (with A overhangs) was added to 1 µl of pCR®2.1-TOPO® vector. In later kits, 1 µl of 1.2 M NaCl, 0.06 M $MgCl_2$ was also added to increase efficiency. The mixture was incubated at room temperature for 5 minutes, then placed on ice. Next, 2 µl of the cloning reaction was added to 50 µl of TOP10 One Shot® Chemically Competent *E. coli* which had been thawed on ice. Cells were incubated on ice for 30 minutes, heat-shocked at 42°C for 30 seconds, and placed on ice for 2 minutes. A 250 µl aliquot of SOC media (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added to the cells and the mixture was incubated at 37°C for 1 hour while shaking. The entire amount was then spread on LB solid medium containing ampicillin (50 µg/ml) and X-gal (50-60 µg/ml) and incubated overnight at 37°C. White colonies were chosen for analysis and all PCR-amplified products were sequenced to ensure polymerase fidelity.

f) Site-directed mutagenesis

Site-directed mutants were made using the MORPH™ Site-Specific plasmid DNA Mutagenesis Kit (5 Prime→3 Prime, Inc.) or the GeneEditor™ *in vitro* Site-Directed Mutagenesis System (Promega). All mutagenic oligonucleotides were 33 nucleotides long with the mismatch nucleotides at the center and were 5'-phosphorylated (Life Technologies or Sigma).

MORPH™ Kit: To denature the double-stranded DNA and anneal the mutagenic oligonucleotide, a mixture containing 1X MORPH annealing buffer, 0.03 pmol target plasmid DNA, and 100 ng of mutagenic oligonucleotide in a total volume of 20 µl was prepared. The DNA was denatured at 100°C for 5 minutes then the tube was placed on ice for 5 minutes. The tubes were then incubated at room temperature for 30 minutes to

allow the mutagenic oligonucleotide to anneal to the correct location on the target plasmid DNA. To synthesize the replacement strand, 8 µl of 3.75X MORPH Synthesis buffer, 3 U of T4 DNA polymerase, and 4 U of T4 DNA ligase were added to the annealing reaction. The tube was incubated at 37°C for 2 hours, then heated at 85°C for 15 minutes to stop the reaction. To digest the methylated, non-mutagenized target plasmid DNA, the mixture was incubated with 1 µl of diluted *Dpn*I (diluted 1:10 with *Dpn*I dilution buffer) at 37°C for 30 minutes, then placed in ice for 5 minutes. To transform the mutagenesis reaction, 200 µl of competent *E. coli* MORPH *mutS* cells were thawed and all of the reaction was added directly to the cells on ice. The cells were incubated on ice for 20 minutes, heat-shocked at 42°C for 2 minutes, and placed at room temperature. All of the transformation was spread onto LB solid medium containing ampicillin (50 µg/ml) and incubated overnight at 37°C.

GeneEditor™: To denature the double-stranded template, a mixture containing 0.5 pmol of template DNA and 2 µl of 2 M NaOH, 2 mM EDTA in a total volume of 20 µl was prepared and incubated at room temperature for 5 minutes. The denatured DNA was precipitated by adding 2 µl of 2 M ammonium acetate (pH 4.6) and 75 µl of 100% ethanol. The solution was placed at -80°C for 30 minutes and centrifuged at 12,000 x *g* for 15 minutes at 4°C. The pellet was washed with 200 µl of 70% ethanol, dried under a vacuum, and resuspended in 100 µl of TE buffer. To anneal the mutagenic oligonucleotide to the denatured double-stranded DNA, a mixture of 10 µl of template DNA (0.05 pmol), 0.25 pmol of the phosphorylated bottom selection oligonucleotide (supplied with kit), 1.25 pmol of the phosphorylated mutagenic oligonucleotide, and 1 X annealing buffer in a total volume of 20 µl was prepared. The annealing reactions were

heated at 75°C for 5 minutes and cooled to 37°C at a rate of 1.5°C per minute. To synthesize the mutant strand, 5 µl of dH_2O, 3 µl of 10 X synthesis buffer, 5-10 U of T4 DNA polymerase, and 1-3 U of T4 DNA ligase were added to the annealing reaction and incubated at 37°C for 90 minutes. To transform the mutagenesis reaction, 100 µl of BMH 71-18 *mutS* competent cells were thawed and transferred to a sterile 13 ml tube, to which 1.5 µl of the mutagenesis reaction was added. The tubes were incubated on ice for 10 minutes, heat-shocked at 42°C for 50 seconds, placed on ice for 2 minutes, 900 µl of LB was added, and cultures were incubated at 37°C for 1 hour. To prepare overnight cultures, 4 ml of LB containing 100 µl of the GeneEditor™ Antibiotic Selection Mix was added to the transformation reaction and incubated overnight at 37°C. The following day, plasmid DNA was isolated from the overnight culture using the Sigma GenElute™ Plasmid Miniprep Kit. Plasmid DNA (1 µl) was then transformed into either JM109 or DH5α competent cells for analysis of mutants.

g) DNA Sequencing

DNA was sequenced by the dideoxy method (Sanger et al., 1977) using the ^{17}Sequencing™ kit from Pharmacia Biotech. Plasmid DNA to be sequenced was isolated from bacteria as described above. To denature the double-stranded template, a mixture of 16 µl plasmid DNA, 16 µl dH_2O, and 8 µl of 2 M NaOH was prepared and incubated at room temperature for 10 minutes. To precipitate denatured DNA, 7 µl of 3 M sodium acetate (pH 4.8), 4 µl of dH_2O, and 120 µl of 100% ethanol were added, the mixture was placed at -80°C for 15 minutes, then centrifuged at 12,000 x *g* for 15 minutes at 4°C. The pellet was rinsed with ice-cold 70% ethanol, decanted, dried under a vacuum and resuspended in 10 µl dH_2O. To 10 µl of the denatured template DNA, 2 µl of annealing

buffer (1 M Tris-HCl pH 7.6, 100 mM $MgCl_2$, 160 mM DTT) and 2 µl of a 5 µM stock of the appropriate primer were added. The mixture was incubated at 65°C for 5 minutes, quickly transferred to a 37°C water bath, incubated for 10 minutes, then placed at room temperature for 5 minutes. To the tube containing the annealed template and primer, 3 µl of Labelling Mix-dATP (1.375 µM each of dCTP, dGTP, and dTTP and 333.5 mM NaCl), 1 µl of [^{35}S]-deoxyadenosine 5'-(α-thio) triphosphate (12.5 µCi/µl, NEN), and 2 µl of diluted T7 polymerase (diluted 1:5 with 20 mM Tris-HCl pH 7.5, 5 mM DTT, 100 µg/ml BSA, 5% glycerol) were added. The labelling reaction was incubated at room temperature for 5 minutes, then 4.5 µl was transferred to each of four microcentrifuge tubes containing 2.5 µl of either 'A' mix-short, 'C' mix-short, 'G' mix-short, or 'T' mix-short. These termination reactions were incubated at 37°C for 5 minutes, then 5 µl of Stop Solution (0.3% bromophenol blue, 0.3% xylene cyanol FF, 10 mM EDTA pH 7.5, 97.5% deionized formamide) was added. The samples were heated at 80°C for 2 minutes, then 3-4 µl was loaded onto a 8% sequencing gel (8% acrylamide/bisacrylamide (29:1), 23% urea, 1 X Tris-borate buffer, 0.05% ammonium persulfate, 0.1% TEMED). Gels were run using a Model S2 sequence apparatus (Life Technologies) at 50 mA for 2-3 hours, placed on a sheet of 3M Whatman filter paper, and dried at 80°C for 1 hour. The dried gel was exposed to X-Omat AR film (Kodak) overnight.

DNA was also sequenced by the Li-Cor automated sequence facility at the National Research Council (Dalhousie University).

h) Amplification of cDNA Library

The cDNA library used to clone the hCPT1 and hCPT1β cDNAs was a SUPERSCRIPT™ Human Brain cDNA Library (Life Technologies), which was

directionally cloned into the *Not*I-*Sal*I region of the eukaryotic expression vector pCMV-SPORT. Through serial dilutions, the titre of the library was determined to be 5.5 x 10^6 cfu/µl. A 100 ml volume of terrific broth (1.2% tryptone, 2.4% yeast extract, 0.4% glycerol, 17 mM KH_2PO_4, 72 mM K_2HPO_4) containing ampicillin at 100 µg/ml was inoculated with 1 µl of the library to give at least 1-2 x 10^6 independent clones. The culture was grown overnight at 30°C, and the absorbance at 600 nm was determined to be 5.0. The entire culture (500 OD units) was centrifuged at 4800 x *g* for 15 minutes at 4°C, decanted, and resuspended in 10 ml Buffer I (15 mM Tris-HCl pH 8.0, 10 mM EDTA) containing RNase A at 100 µg/ml. A 10 ml volume of Buffer II (0.2 M NaOH, 1% SDS) was added and the tube was inverted to mix, and incubated at room temperature for 5 minutes. Next, 10 ml of ice-cold 7.5 M ammonium acetate was added and the mixture was incubated on ice for 10 minutes, and centrifuged at 3000 x *g* for 15 minutes at 4°C. The supernatant was transferred to a new tube, an equal volume of cold isopropanol was added, then centrifuged at 3000 x *g* for 15 minutes at 4°C. The resulting pellet was resuspended in 1 ml of Buffer I and transferred to a microcentrifuge tube. The solution was centrifuged for 1 minute at 4°C and the supernatant was transferred to a fresh tube, which was incubated at 37°C for 30 minutes, then at 65°C for 5 minutes. A 500 µl aliquot was transferred to each of two separate microcentrifuge tubes and 400 µl of phenol:$CHCl_3$:isoamylalcohol (25:24:1) was added to each tube. After vortexing, the tubes were centrifuged for 5 minutes at room temperature and the upper aqueous layer was transferred to a new tube. The phenol:$CHCl_3$:isoamylalcohol wash was repeated 3 times, after which an equal volume of cold isopropanol was added, and the mixture was vortexed and centrifuged for 15 minutes at 4°C. The pellet was washed with 500 µl of

cold 70% ethanol, decanted, dried at room temperature, and resuspended in 200 µl of sterile TE buffer. The DNA concentration was determined by absorbance at 260 nm to be 7.8 µg/µl.

2. Yeast protocols

a) High efficiency transformation of yeast

Plasmid DNA was introduced into yeast by the lithium acetate method (Gietz et al., 1992). Yeast were grown overnight in 1 ml YEPD medium at 30°C or 25°C (temperature-sensitive strains), diluted to an absorbance of 0.25 in 10 ml fresh YEPD, and grown until the absorbance at 600 nm was between 0.5-0.8 (approximately 4 hours). Cells were pelleted at 3000 rpm for 5 minutes and washed once with 1 ml sterile dH_2O. The pellet was resuspended in 50 µl of 0.1 M lithium acetate, 1 X TE buffer and transferred to microcentrifuge tubes containing 50 µg denatured herring sperm DNA (denatured by boiling for 10 minutes and placed on ice) and 1 µg of plasmid DNA. Next, 300 µl of 40% PEG 3400, 0.1 M lithium acetate, 1 X TE was added and tubes were incubated at 30°C for 30 minutes. Cells (with the exception of temperature-sensitive strains) were heat-shocked at 42°C for 15 minutes, centrifuged at 12,000 x *g* for 15 seconds, and resuspended in 200 µl of 1 X TE buffer. The entire amount was spread onto SD solid medium containing the appropriate nutrients for plasmid selection and incubated for 3-4 days at either 30°C or 25°C, depending on the yeast strain.

b) Subcellular fractionation of yeast

Yeast cells were grown overnight at 30°C (25°C for temperature-sensitive strains) in 5 ml of SD containing the appropriate nutrients for plasmid maintenance. The next day, the yeast cells were back-inoculated in 200 ml of medium and allowed to grow to mid-

log phase (0.6-0.9 absorbance at 600 nm). Cultures were centrifuged at 1000 x *g* for 10 minutes, decanted and resuspended in 25 ml of ice-cold GTE buffer (20% glycerol, 50 mM Tris-HCl pH 7.4, 1 mM EDTA). Cells were centrifuged at 1000 x g for 10 minutes, decanted and resuspended in 0.7 ml GTE buffer, which was then transferred to a test tube containing approximately 0.5 ml of 0.5-mm glass beads (Sigma). This mixture was vortexed at high speed 5 times for 30 seconds each with 30 seconds on ice in between each mixing. The resulting homogenate was transferred to a new tube and the beads were rinsed twice with 0.5 ml GTE buffer, with each rinse being added to the homogenate. To pellet unbroken cells, nuclei, and mitochondria, the homogenate was centrifuged at 16,000 x *g* for 15 minutes at 4°C. The supernatant was transferred to an ultracentrifuge tube and spun at 450,000 x *g* for 15 minutes at 4°C. The cytosol (supernatant) was transferred to a new tube and the microsomes (pellet) were resuspended by Dounce homogenization in 0.5 ml GTE buffer. All fractions were aliquoted and stored at -80°C.

c) Metabolic labelling and yeast lipid extraction (Folch)

As an *in vivo* measure of PC and PE biosynthesis, yeast cells containing the appropriate expression plasmids were labelled with [^{14}C]choline and ethanolamine precursors. Yeast cells were grown overnight at 30°C (25°C for temperature-sensitive strains) in 3 ml of SD medium containing the appropriate nutrients for plasmid maintenance. This medium is both choline- and ethanolamine-free. The overnight culture was diluted to an absorbance (at 600 nm) of 0.2 in 3 ml fresh medium and grown to an absorbance of between 0.35 and 0.75 (approximately 4 hours). Because yeast cells do not readily take up ethanolamine in the presence of ammonium sulfate, the cultures destined for ethanolamine labelling were centrifuged at 2500 rpm for 5 minutes and washed twice

with 5 ml of SD medium without ammonium sulfate. Cell pellets were resuspended in 3 ml of SD without ammonium sulfate but containing the appropriate nutrients required for plasmid maintenance. *Ethanolamine labelling*: A 10 µl aliquot of [^{14}C]ethanolamine hydrochloride (ICN) was added directly to a 3-ml growing culture. *Choline labelling*: A 30 µl aliquot of 1 mM choline, together with 3 µl of [^{14}C]choline chloride [methyl-^{14}C] (NEN) was added directly to a 3 ml growing culture. Cultures were routinely labelled at 30°C (or 25°C for temperature-sensitive strains) for 1 hour (unless otherwise indicated) and lipids were extracted by the Folch method (Folch et al., 1957).

Subsequent to metabolic labelling of yeast cultures, lipids were extracted by a modified Folch method (Folch et al., 1957). Cultures were centrifuged at 3300 rpm for 5 minutes and pellets were washed twice with 1 ml of water. Next, 1 ml of $CHCl_3/CH_3OH$ (1/1, v/v) was added, tubes were vortexed and cells were transferred to a 2-ml bead beater vial which was 1/8 filled with 0.5-mm acid washed glass beads (Sigma). Yeast cells were disrupted in a BioSpec Multi bead beater for 1 minute at 4°C. The cell extract was transferred to a test tube and the beads were rinsed once with 1 ml of $CHCl_3/CH_3OH$ (2/1, v/v). This rinse was added to the first extract and 0.5 ml of $CHCl_3/CH_3OH$ (2/1, v/v) was added to the mixture. At this point, a 100-µl sample was removed to determine total cell uptake. To facilitate phase separation, 0.5 ml of $CHCl_3$ and 1.5 ml of water were added, tubes were vortexed, and centrifuged at 2500 rpm for 10 minutes. The upper aqueous phase was transferred to a new tube, the middle protein layer was aspirated off, and the lower organic phase was transferred to a new tube.

To measure alterations in phospholipid synthesis, yeast cells were labelled with inorganic phosphorus-32. Yeast cells of strain CTY434 were grown overnight at 25°C in

minimal medium containing the appropriate nutritional supplements. Cells were back-inoculated in minimal medium containing the required supplements and grown at 25°C. When cells reached early log phase, inorganic phosphorus-32 (2.5 mCi) was added to 20 ml of culture. Samples of 5 ml were removed at various time points and centrifuged at 3000 x *g* for 5 minutes to pellet cells, which were then washed twice with 5 ml of ice-cold water, resuspended in 1 ml of $CHCl_3/CH_3OH$ (1/1, v/v), and transferred to 2-ml screw-capped tubes containing 0.5 g of 0.5-mm glass beads. For experiments requiring extraction of di8:0 phosphatidic acid, perchloric acid was added to the extraction mixture at 0.7% (v/v). Cells were disrupted using a BioSpec Multi bead beater for 1 minute at 4°C. The beads were washed with 1.5 ml of $CHCl_3/CH_3OH$ (2/1, v/v) and 0.5 ml of $CHCl_3$ and 1.5 ml of H_2O were added to the combined homogenate. Tubes were vortexed and centrifuged at 2000 x *g* for 10 minutes to facilitate phase separation. The organic phase was washed once with an equal volume of 40% CH_3OH (v/v) and a sample of the organic phase was dried under nitrogen in the presence of phospholipid standards. Phospholipids were separated by two-dimensional TLC with $CHCl_3/CH_3COCH_3/CH_3OH/CH_3COOH/H_2O$ (60:24:12:12:6, v/v) in the first dimension and $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (50:37.5:3.5:2, v/v) in the second dimension. Plates were exposed to x-ray film for 24-48 hours and subsequently stained with iodine vapor. Iodine-stained spots corresponding to the mobility of known standards were scraped into scintillation vials and counted.

d) Lipid phosphorus determination

To determine phospholipid mass, the method of Ames and Dubin (Ames and Dubin, 1960) was used. Generally, 600 µl of lipid sample extracted from a 3-ml yeast culture

was dried down under nitrogen gas and standards were prepared by adding between 0-60 nmol of 1 mM potassium dihydrogen phosphate to separate tubes. Water was added to all tubes to a volume of 300 µl and 150 µl of perchloric acid was added. All tubes were incubated overnight at 160°C with marbles on top of tubes to prevent evaporation. Next, 700 µl of dH_2O was added and tubes were incubated at room temperature for 20 minutes. 500 µl of 0.9% ammonium molybdate was added, the tubes were thoroughly mixed, and 150 µl of freshly prepared 10% ascorbic acid was added. Tubes were mixed and incubated at 45°C for 30 minutes. Absorbance was read at 820 nm.

e) Suppression of *cpt1*-mediated bypass of *sec14*^{ts}

Cells of strain CTY434 (*sec14*^{ts} *cpt1::leu2*) containing various plasmids were grown overnight at 25°C in SD medium containing the appropriate nutrients for plasmid maintenance. Optical densities were measured at 600 nm and each strain was diluted to the identical cell number. Serial dilutions of 1:10, 1:100, and 1:1000 were made in sterile dH_2O and 1 µl of each dilution was spotted onto SD solid medium containing the appropriate nutrients for plasmid maintenance ± 200 µM di8:0 DAG (diacylglycerol stock was dried under nitrogen gas to evaporate $CHCl_3$, resuspended in sterile dH_2O by probe sonication, and added to the medium after allowing the autoclaved medium to cool to 55°C). Plates were incubated at 25°C or 37°C for 4-7 days and the amount of growth was assessed.

f) Invertase secretion

To measure invertase secretion, yeast cells were grown overnight at 25°C in 3 ml of YEPD medium. The overnight culture (0.5 - 1 ml) was used to inoculate 4.5 - 4 ml of fresh YEPD (2% glucose). Cultures were grown at 25°C for 2 hours, then centrifuged at

2500 rpm for 5 minutes to pellet yeast. Pellets were washed twice with 5 ml sterile dH_2O and resuspended in 5 ml of YEPD containing 0.1% glucose. Cultures were subsequently grown at 37°C for 2 hours to impose the temperature-sensitive phenotype. Invertase secretion was measured using the method described by Goldstein and Lampen (Goldstein and Lampen, 1975) and Bankaitis *et al.* (Bankaitis et al., 1989). Between 1-5 ml of the culture was centrifuged at 2500 rpm for 5 minutes, washed twice with 1 ml of 10 mM sodium azide, and resuspended in 500 µl of sodium azide. Next, 250 µl of cells were transferred into each of two microcentrifuge tubes. To one tube, 250 µl of 10 mM sodium azide was added (-) and to the other tube, 250 µl of 10 mM sodium azide, 0.2% Triton X-100 was added (+). (+) tubes were vortexed and placed at -80°C for 10 minutes to break open cells, while the absorbance of the (-) tubes was determined at 600 nm. Both (+) and (-) tubes were set up in triplicate to contain an appropriate amount of cells, based on the above absorbance, in a total volume of 200 µl of 0.1 M sodium acetate (pH 5.1). Tubes were placed at 30°C and 50 µl of 0.5 M sucrose was added. After incubating samples at 30°C for exactly 30 minutes, 300 µl of 0.2 M potassium phosphate (pH 7) containing 10 mM *N*-ethylmaleimide (NEM) was added to stop the reaction, and samples were boiled for 3 minutes. To develop the assay, tubes were equilibrated at 30°C, and 2 ml of glucostat reagent (0.1 M potassium phosphate, pH 7, 4.35 U glucose oxidase, 2.5 µg/ml peroxidase, 0.1 mM NEM, 150 µg/ml *O*-dianisidine) was added. After incubating samples at 30°C for exactly 30 minutes, 2 ml of 6 N hydrochloric acid was added to stop the reaction. Absorbance at 540 nm was measured and the invertase secretion index of each sample was determined by dividing external invertase (-) by total invertase (+).

3. Mammalian Cell Culture

a) Lipofectamine transfection of cultured cells

Chinese hamster ovary cells (CHO-K1) were transiently transfected using LIPOFECTAMINE™ Reagent (Life Technologies). Cells were seeded at a density of 5 x 10^5 cells per 60-mm dish in DMEM containing 5% (v/v) FCS and proline at 33 μg/ml (CKO-K1 medium). Cells were grown overnight at 37°C in an atmosphere of 5% CO_2 to a confluence of 50-80%. Solution A was prepared by diluting 3 μg of Qiagen-purified plasmid DNA into 200 μl of DMEM for each transfection. Solution B was prepared by diluting 10 μl LIPOFECTAMINE™ Reagent into 200 μl of DMEM for each transfection. The two solutions were combined (400 μl per transfection) and incubated at room temperature for 45 minutes to allow DNA-liposome complexes to form, during which time the cells were rinsed once with 2 ml of DMEM. For each transfection, 1.6 ml of DMEM was added to the tube containing the complexes, mixed gently, and overlaid onto the rinsed cells. The cells were incubated with the complexes at 37°C in an atmosphere of 5% CO_2 for 5-8 hours. Following this incubation, 2 ml of DMEM containing 10% FCS and proline (33 μg/ml) was added without removing the transfection mixture. The cells were incubated overnight at 37°C (5% CO_2) and 18-24 hours following the start of transfection, the medium was replaced with 3 ml fresh CHO-K1 medium and grown to the desired confluence.

b) Calcium phosphate transfection

To stably transfect CHO-K1 cells, the calcium phosphate method was used. Cells were seeded at a density of 5 x 10^5 per 100-mm dish in CHO-K1 medium. Solution A was prepared by adding 0.5 ml of 2X HEPES-buffered saline, pH 7.05 (HBS: 280 mM NaCl, 10 mM KCl, 1.5 mM $Na_2HPO_4.2H_2O$, 12 mM dextrose, 50 mM HEPES) to one

tube. Solution B was prepared by combining 10 μg of plasmid DNA (in a total of 440 μl of dH_2O) and 62 μl of 2 M calcium chloride ($CaCl_2$) in a separate tube. Solution B was added slowly to solution A while bubbling air through solution A with a 1-ml pipette to mix and the combined solutions were incubated at room temperature for 45 minutes. The medium was aspirated from the cells and 1 ml of the combined solution was added. The dish was swirled gently, incubated at 37°C for 10 minutes, swirled gently again, and incubated at 37°C for 10 minutes. Then, 10 ml of CHO-K1 medium was added to the dish without removing the transfection mixture and cells were incubated at 37°C for 5 hours. The medium was aspirated off, cells were rinsed twice with 10 ml of PBS, and 8 ml of fresh CHO-K1 medium was added. The cells were incubated overnight at 37°C, then the medium was replaced with 8 ml of CHO-K1 medium containing G418 at 500 μg/ml to start selection of stably transfected clones. Subsequently, the medium was replaced every 2-3 days with 8 ml of fresh CHO-K1 medium containing G418 (500 μg/ml) to select for clones that were stably transfected with the desired plasmid.

c) Dilution cloning

Single colonies of stably transfected CHO-K1 cells were selected by dilution cloning. The 100-mm dishes were rinsed once with 8 ml of PBS and 0.9 ml of trypsin was added to release cells from the bottom of the dish. Then, 7 ml of CHO-K1 medium was added to the cells to dilute the trypsin and this was added to 13 ml of CHO-K1 medium in a 50-ml tube. A 1-ml sample was removed and the remaining 19 ml was centrifuged for 10 minutes to pellet cells. The 1-ml sample was mixed well and the number of cells was determined using a hemocytometer. The cell pellet was resuspended in DMEM to give 7 x 10^6 cells/ml and serially diluted to 7 cells/ml in CHO-K1 medium

containing G418 (500 μg/ml). Aliquots of 200 μl were pipetted into 48-well plates and incubated at 37°C in an atmosphere of 5% CO_2 for 3-4 days. Those wells containing 1 cell per well were incubated for 7 days and 100 μl of CHO-K1 medium containing G418 (500 μg/ml) was added. Medium was replaced as required and then cells were transferred to larger dishes for screening. Positive colonies were identified by Western blotting with the T7-HRP conjugated antibody.

d) Harvesting cells

To harvest cultured cells, medium was aspirated from dishes and cells were washed once with ice-cold PBS. Ice-cold PBS (3 ml) was added, and cells were scraped into a sterile tube and centrifuged at 3000 rpm for 3 minutes. The cell pellet was resuspended in 1 ml of cold homogenization buffer (10 mM HEPES pH 7.4, 50 mM KCl, 1 mM EDTA) and homogenized by passing through a syringe with 23 gauge needle 20 times. The homogenate was centrifuged for 15 seconds at 4°C to pellet unbroken cells, and the supernatant was transferred to an ultracentrifuge tube and centrifuged at 450,000 x *g* for 15 minutes at 4°C. The cytosol (supernatant) was transferred to a new tube and the membrane-enriched fraction (pellet) was resuspended by Dounce homogenization in 300-500 μl homogenization buffer. All fractions were aliquoted and stored at -80°C.

e) Immunofluorescence

T⁻. ER, Golgi Apparatus, Nuclear membrane staining: All cells used for immunofluorescence were grown on glass coverslips in 60-mm dishes at a density of 2 x 10^5 cells per dish. Cells were fixed in 3% (v/v) formaldehyde in 10 mM sodium phosphate (pH 7.4), 225 mM NaCl, 2 mM $MgCl_2$ (PBS-B) for 15 minutes at room temperature. The coverslips were washed twice at room temperature with PBS-B

containing 5 mM ammonium chloride for 5 minutes each wash, and rinsed once with PBS-B for 5 minutes. Cells were then permeabilized by adding PBS-B containing 0.05% Triton X-100 and incubating at 4°C for 15 minutes, followed by incubation at room temperature for 15 minutes. The coverslips were washed twice with PBS-B containing 1% fatty acid free BSA (PBS/BSA) for 5 minutes per wash, then once for 15 minutes. Cells were treated with an appropriate dilution of primary antibody in PBS/BSA for 1 hour at room temperature. The dilutions used are indicated in the appropriate figure legends. The coverslips were then washed twice with PBS/BSA for 5 minutes each. With the exception of cells incubated with LcH-lectin (which was conjugated to FITC), this was followed by incubation with the secondary antibody at a dilution of 1:4000 in PBS/BSA for 1 hour at room temperature. A number of secondary antibodies were used, including FITC-labelled goat anti-mouse (GAM-FITC), Texas Red X-labelled goat anti-mouse (GAM-TxRed), Texas Red X-labelled goat anti-rabbit (GAR-TxRed), and FITC-labelled goat anti-rabbit (GAR-FITC). Subsequently, cells were washed twice with PBS/BSA for 5 minutes each and coverslips were mounted on slides with 2.5% (w/v) 1,4-diazadicyclo-[2.2.2]-octane in 50 mM Tris-HCl (pH 9.0) and 90% (v/v) glycerol (Ridgway et al., 1992). Fluorescence microscopy was performed on a Zeiss microscope.

Mitochondrial staining: To stain the mitochondria, cells were grown on glass coverslips as described above. Prior to fixing, the medium was aspirated from the cells and replaced with fresh CHO-K1 medium containing 200 nM MitoTracker® Red CMXRos (Molecular Probes). The cells were incubated with the dye for 45 minutes at 37°C, then washed twice with warm PBS. Cells were fixed and mounted as described above.

GFP Fluorescence: To visualize cells that were transiently transfected with GFP fusion

proteins. cells grown on glass coverslips were washed three times with 58 mM Na_2HPO_4, 17 mM NaH_2PO_4, 68 mM NaCl (PBS-C, pH 7.4), fixed by incubating with PBS-C/4% formaldehyde for 30 minutes at room temperature, washed twice with PBS-C, and coverslips were mounted as described above.

4. Other protocols

a) GeneTrapper® cDNA Positive Selection System

To clone the hCPT1 and hCPT1β cDNAs from the brain cDNA library, the GeneTrapper® cDNA Positive Selection System was used (Life Technologies). The oligonucleotide used in this experiment was PAGE purified (to get rid of aborted synthesis products) and corresponded to the most 5' region of the known partial hCPT1 sequence (5'-TATCGGCGGGTGGAGCACCGCTACAGC). To biotinylate the oligonucleotide, a mixture of 3 µg of the oligonucleotide, 5 µl of 5X TdT (terminal deoxynucleotidyl transferase) buffer, 5µl of biotin-14-dCTP, and 2 µl of TdT in a total volume of 25 µl was prepared and incubated at 30°C for 1 hour. To precipitate the biotinylated oligonucleotide, 1 µl of glycogen (20 µg/µl), 26 µl of Tris-HCl (pH 7.5), and 120 µl of ethanol were added, the solution was placed at -80°C for 30 minutes, then centrifuged at 14,000 x *g* for 30 minutes at 4°C. The pellet was washed twice with 200 µl of 70% ethanol, allowed to dry, and resuspended in 20 µl TE buffer.

To generate single-stranded DNA, phage F1 endonuclease (Gene II) and Exonuclease III (Exo III) were used. A mixture of 10 µg of double-stranded brain cDNA library, 2 µl of 10 X Gene II buffer, and 1 µl of Gene II was prepared in a total volume of 20 µl and incubated at 30°C for 25 minutes. The mixture was incubated at 65°C for 5 minutes, placed on ice for 1 minute, and a 1-µl sample was removed for analysis. Next, 2

µl of Exo III was added and incubated at 37°C for 1 hour. An equal volume of phenol:$CHCl_3$:isoamylalcohol (25:24:1) was added, vortexed, and centrifuged for 5 minutes at room temperature. The upper (aqueous) phase was transferred to a new tube and stored at 4°C until the digestion was confirmed by agarose gel electrophoresis.

To capture the desired cDNA, 6 µl of 4 X Hybridization buffer was added to 17 µl of Gene II/Exo III-treated DNA, and the mixture was denatured at 95°C for 1 minute and placed on ice for 1 minute. One microliter of diluted biotinylated oligonucleotide (diluted to 10 ng/µl in TE buffer) was added to the denatured DNA and the tube was incubated at 37°C for 1 hour. The streptavidin paramagnetic beads were prepared by transferring 45 µl to a microcentrifuge tube and inserting the tube into the magnet for 2 minutes. The supernatant was removed and 100 µl of TE buffer was added to the beads, mixed, and inserted into the magnet for 2 minutes. The supernatant was removed and the beads were resuspended in 30 µl TE buffer. The prepared paramagnetic beads were transferred into the single-stranded DNA/biotinylated oligonucleotide hybridization mixture following the incubation at 37°C and the suspension was incubated at room temperature for 30 minutes. The tube was inserted into the magnet for 2 minutes, the supernatant was removed, and 100 µl Wash buffer was added to the beads. The tube was inserted into the magnet for 2 minutes, the supernatant removed, and this step was repeated once more. Next, 100 µl of Wash buffer was added, the suspension was transferred to a new tube and the tube was inserted into the magnet for 5 minutes. This step was repeated once more, then 20 µl of 1 X Elution buffer was added and incubated at room temperature for 5 minutes. The tube was inserted into the magnet for 5 minutes and the supernatant (containing the captured cDNA clone) was transferred to a new tube. The beads were

resuspended in 15 µl of TE buffer and the tube was inserted into the magnet for 5 minutes. The supernatant was combined with the first supernatant and 1 µl of glycogen, 18 µl of 7.5 M ammonium acetate, and 135 µl of ethanol was added to precipitate the DNA. The mixture was placed on ice for 10 minutes and centrifuged at 14,000 x *g* for 30 minutes at 4°C. The pellet was washed with 100 µl of 70 % ethanol, allowed to dry, and resuspended in 5 µl of TE buffer. To repair the captured cDNA, 11 µl of dH_2O, 1 µl of non-biotinylated oligonucleotide (50 ng), 0.5 µl of 10 mM dNTP mix, 2 µl of 10 X repair buffer, and 0.5 µl of repair enzyme were added to the captured cDNA. The mixture was incubated at 90°C for 1 minute, 55°C for 30 seconds, and 70°C for 15 minutes. To precipitate DNA, 1 µl of glycogen, 11 µl of 7.5 M ammonium acetate, and 90 µl of ethanol were added, and the tubes were placed on ice for 10 minutes and centrifuged at 14,000 x *g* for 30 minutes at 4°C. The pellet was washed with 100 µl of 70% ethanol, allowed to dry, and resuspended in 10 µl of TE buffer. To electroporate the repaired DNA, 2 µl of DNA was placed into a microcentrifuge tube and 40 µl of ElectroMax DH10B cells (Life Technologies) were added. Cells were electroporated using the BioRad Gene Pulser at 1.61 kV, 100 ohms, and 25 µF, and then 1 ml of SOC media was added and the diluted cell suspension was transferred to a sterile 13-ml tube and incubated at 37°C for 1 hour. Cells were spread onto LB solid medium containing ampicillin at 100 µg/ml and incubated overnight at 37°C. Positive clones were identified by PCR using primers specific to the original EST.

b) Northern blot analysis

Preparation of probes: The 1248-base pair open reading frame of hCEPT1 was amplified by PCR from a Bluescript plasmid containing the cDNA for hCEPT1 (pAH1),

using the oligonucleotide primers 5'-GCGGGATCCATGAGTGGGCATCGATCAACA (forward) and 5'-GCGGTCGACTTAATGATTAGAATGAGCTGT (reverse). The PCR product was purified using a PCR-Select III spin column (5'→3') to remove unused reagents, then GeneCleaned (Bio101) to separate it from non-specific PCR bands. The DNA was diluted to a concentration of 25 ng/μl for use with the Multiprime Labelling Kit (Amersham). For positive controls, a 2.0-kbp human β-actin cDNA supplied with the Northern blots was used. The first step in synthesizing the hCEPT1 and β-actin probes was to denature 50 ng of DNA by incubating in a boiling water bath for 5 minutes and placing it immediately on ice. Then, 20 μl of Multiprime buffer solution (dATP, dGTP, and dTTP in a concentrated buffer solution containing Tris-HCl [pH 7.8], $MgCl_2$, and 2-mercaptoethanol), 10 μl of primer solution (random hexanucleotides in an aqueous solution containing nuclease-free BSA), 10 μl of [α-^{32}P]dCTP (3000 Ci/mmol), 4 μl Klenow (1 unit/μl), and water to 100 μl were added to the denatured DNA on ice. The reaction was incubated at 37°C for 30 minutes, then purified by passing through a PCR Select-I spin column (5'→3').

The 1218-base pair open reading frame of hCPT1 was amplified by PCR from a pCMV-SPORT vector containing the cDNA for hCPT1 (GT13) using the oligonucleotide primers 5'-GCCAGATCTATGGCGGCAGGCGCCGGGGCC (forward) and 5'-GCCGTCGACTCAATCCATGTTATTCTGATG (reverse). The PCR product was purified by GeneClean and diluted to a concentration of 25 ng/μl for use with the Random Primers DNA Labeling System (Life Technologies). First, 50 ng of DNA was diluted into 20 μl of water and denatured by incubating in a boiling water bath for 5 minutes and placing immediately on ice. Then, 4 μl each of dATP, dTTP, and dGTP (0.5

mM in 1mM Tris-HCl [pH 7.5]), 30 µl of Random Primers Buffer Mixture (0.67 M HEPES, 0.17 M Tris-HCl, 17 mM $MgCl_2$, 33 mM 2-mercaptoethanol, 1.33 mg/ml BSA, 18 OD_{260} units/ml oligodeoxyribonucleotide primers (hexamers), pH 6.8), 10 µl of [α-^{32}P]dCTP (3000 Ci/mmol), 2 µl of Klenow (3 units/µl), and water to 100 µl was added to the denatured DNA on ice. The reaction was incubated at 25°C for 1 hour, then purified by passing through a PCR Select-I spin column. The activity of all cDNA probes was determined by scintillation counting.

Northern Blot: For all Northern blot applications, ExpressHyb™ (Clontech) was used. ExpressHyb™ is a hybridization solution that has been optimized for Northern and Southern analyses on positively charged membranes. The ExpressHyb™ was warmed at 68°C and 5 ml was added to each of 2 blots. The membranes were prehybridized at 68°C for 30 minutes with continuous shaking. Meanwhile, the probe was denatured by incubating in a boiling water bath for 5 minutes and placing immediately on ice. A total of 1 x 10^7 dpm of the denatured probe was added to 5 ml (per blot) of fresh ExpressHyb™ and the prehybridization solution was replaced with the fresh solution containing the radiolabelled probe. The blots were incubated at 68°C for 1 hour with continuous shaking, then washed for 30-40 minutes at room temperature with 2X SSC (0.3 M NaCl, 0.03 M sodium citrate [pH 7.0]), 0.05% SDS, replacing the wash solution several times. The blots were washed with 0.1X SSC, 0.1% SDS for 40 minutes at 50°C, changing the solution once, then covered in plastic wrap and exposed to x-ray film. To remove the probe from the blots, 1 L of sterile water containing 0.5% SDS was heated to boiling and the blots were incubated in the solution for 10 minutes. The solution was allowed to cool for 10 minutes and the blots were air-dried and stored in a plastic bag at -

20°C.

c) Lowry protein determination

Protein concentrations were determined by the method of Lowry (Lowry et al., 1951). Both samples and standards (BSA at 1 mg/ml) were set up in test tubes to which 100 µl of 2% sodium cholate was added, followed by dH_2O to a total volume of 1 ml. Next, equal amounts of 1% copper sulfate and 2% potassium sodium tartarate were mixed and added to 2% sodium carbonate (in 0.1 M sodium hydroxide) in a 1:50 ratio. Four milliliters of this mixed solution was added to each tube and tubes were incubated at room temperature for 10 minutes. Folin's phenol reagent (BDH) was diluted 2:1 with dH_2O and 0.5 ml was added to each tube and mixed immediately. The tubes were incubated at 60°C for 10 minutes, allowed to cool to room temperature, and their absorbance at 730 nm was determined. Protein concentrations were determined through comparison with the standard curve.

d) SDS-PAGE and Western Blot Analysis

Equal amounts of protein were separated on the indicated percentage of acrylamide by SDS-PAGE along with pre-stained markers (Benchmark, Life Technologies). Proteins were transferred to PVDF membranes in transfer buffer (48 mM Tris base, 39 mM glycine, 20% CH_3OH, 0.0375% SDS) overnight at 30 volts. The PVDF membrane was blocked for 1 hour in phosphate-buffered saline (PBS: 140 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4, 1.75 mM KH_2PO_4, pH 7.4) containing 0.05% Tween 20 (PBST) and 5% skim milk powder and washed 3-5 times in PBST.

For conjugated antibodies, membranes were incubated for 1 hour in PBST (± 5% skim milk powder) containing an appropriate dilution of the antibody. T7-HRP was

added at a 1:5000 dilution. Membranes were then washed 6 times for 15 minutes per wash in PBST. Proteins were detected using the ECL kit from Amersham and membranes were exposed to X-Omat Blue film (Kodak).

For monoclonal antibodies that were not directly conjugated to HRP, membranes were incubated for 1 hour in PBST (± 5% skim milk powder) containing an appropriate dilution of antibody and washed six times in PBST. Membranes were then incubated for 1 hour in PBST containing a goat anti-mouse secondary antibody conjugated to horseradish peroxidase at a 1:10,000 dilution (GAM-HRP), followed by 6 washes in PBST. As with the conjugated antibodies, proteins were detected using the ECL kit from Amersham and membranes were exposed to X-Omat Blue film.

e) *In vitro* transcription/translation

To determine if an expression plasmid could synthesize protein *in vitro* the TnT® Quick Coupled Transcription/Translation System (Promega) was used and the protocol was followed as described in the manual. A mixture containing 1 µg of the DNA template, 2 µl of [^{35}S]methionine (Easy Tag Express [^{35}S] Protein Labelling Mix, 11mCi/ml, NEN), 40 µl of TnT® Quick Master Mix, and dH_2O to 50 µl was prepared and incubated at 30°C for 90 minutes. After the incubation, 5 µl of the reaction was removed and added to 5 µl of 2X sample buffer. The samples were incubated at 37°C for 30 minutes and analyzed on a 10% SDS-polyacrylamide gel. The gel was separated from the apparatus and fixed in 50% CH_3OH, 10% glacial acetic acid for 30 minutes at room temperature. The gel was placed on a sheet of Whatman® 3MM filter paper and dried at 80°C for 30-90 minutes on the DrygelSr. slab gel dryer (Model SE1160, Hoefer Scientific Instruments). The gel was exposed to X-Omat Blue film (Kodak) for 1-30

days.

f) Enzyme Assays

i) CPT/EPT (0.004% Tween 20)

A number of methods were employed to assay cholinephosphotransferase (CPT) and ethanolaminephosphotransferase (EPT) activity in microsomal membranes. The assay used, unless otherwise indicated, was an emulsion-based assay (O et al., 1989), whereby 100 nmol of diradylglycerol or ceramide was dried down under nitrogen gas and resuspended in 25 µl of 0.015% (w/v) Tween 20 by sonicating 4 times for 30 seconds. Unless otherwise indicated, the reaction mixture contained 100 mM Tris-HCl (pH 8.0), 20 mM $MgCl_2$, 1 mM EDTA, 1 mM diradylglycerol or ceramide (final Tween 20 concentration of 0.004%, w/v), and 10-25 µg of microsomal protein in a total volume of 100 µl. Components were incubated at room temperature for 5 minutes to allow the lipids to be incorporated into the microsomal membranes. The reaction was initiated by the addition of [^{14}C]CDP-choline or [^{14}C]CDP-ethanolamine (0.4 mM, 2000 dpm/nmol) and assays were incubated at 37°C for 15 minutes. The reaction was terminated by the addition of 3 ml $CHCl_3/CH_3OH$ (2/1, v/v) and 1.5 ml 0.9% (w/v) KCl (McMaster et al., 1996). Tubes were vortexed and phase separation was facilitated by centrifugation at 2000 x *g* for 10 minutes. The aqueous phase was aspirated off and the organic phase was washed twice with 2 ml 40% CH_3OH/H_2O (v/v). An sample of the organic phase was dried in a scintillation vial and radioactivity was determined. A variation of this method also delivered lipids in a lipid emulsion of either 0.1% Tween 20 or 0.1% deoxycholate (w/v) as the final detergent concentration (Sleight and Kent, 1980), but otherwise assay

conditions were identical.

ii) CPT/EPT (Sodium cholate mixed micelle)

A mixed micelle assay for the determination of CPT and EPT activity derived from hCEPT1 was developed in our laboratory that used sodium cholate as the vehicle by which the lipid substrate was delivered. The assay mixture contained 50 mM Tris-HCl (pH 8.0), 20 mM $MgCl_2$, 20% glycerol, 10 mol% DAG and 10 mol% PC in 1% sodium cholate, using 10-25 µg of membrane protein as the enzyme source. The assay mix was incubated at 25°C for 5 min to allow mixed micelle formation, and the assay was initiated by the addition of [^{14}C]CDP-choline or [^{14}C]CDP-ethanolamine (0.4 mM, 2000 dpm/nmol) and incubated at 25°C for 20 minutes. The assay was terminated by the addition of 3 ml $CHCl_3/CH_3OH$ (2/1, v/v) and 1.5 ml 0.9% (w/v) KCl to each 0.1-ml assay (McMaster et al., 1996). Tubes were vortexed and phase separation facilitated by centrifugation at 2000 x *g* for 10 minutes. The aqueous phase was aspirated off and the organic phase was washed twice with 2 ml 40% CH_3OH. A sample of the organic phase was dried in a scintillation vial and radioactivity was determined by scintillation counting.

iii) Mammalian CPT (PC liposome)

The highest reported specific activity of CPT in mammalian microsomes was determined using a PC liposome assay, in which diacylglycerol is cosonicated with Tween 20 and phospholipids (Cornell, 1992; Miller and Weinhold, 1981). The diacylglycerol substrate was prepared by evaporation of the solvent from 5 mg of DAG,

followed by the addition of 10 µl of Tween 20 from a 25 mg/ml aqueous stock. The mixture was vortexed vigorously, then 0.5 ml of a 50 mg/ml stock (in water) of PC was added to the DAG-Tween mixture. The entire mixture was sonicated for approximately 5 minutes using a probe sonicator, with the sample immersed in ice. The assay mixture contained 50 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 0.5 mM EGTA, 0.4 mM [methyl-^{14}C]CDP-choline (specific radioactivity of 0.5), 2.4 mM *sn*-1,2-DAG emulsion, and 30 µg of microsomes in a total volume of 50 µl. The tubes were incubated at 37°C for 15 minutes and the reaction was terminated by the addition of 1.5 ml of $CH_3OH/CHCl_3$ (2:1, v/v). The ^{14}C-labelled PC was extracted by the addition of 0.3 ml of water, 0.5 ml of $CHCl_3$, and 0.5 ml of water in sequence, with thorough vortexing after each addition. The tubes were centrifuged at 2500 rpm for 5 minutes to separate phases, the upper (aqueous) layer was aspirated off, and the lower (organic) phase was washed with 2 ml of CH_3OH/water/$CHCl_3$ (48:47:3, v/v). A sample of the washed organic phase (0.5 ml) was transferred to a scintillation vial, the solvent was evaporated at 75°C, and the radioactivity was determined by scintillation counting.

iv) Yeast CPT (Triton X-100 mixed micelle)

Yeast CPT activity was determined in microsomes using a mixed micelle assay described previously that results in 10 mol% PC and 10 mol% DAG per assay (Hjelmstad and Bell, 1992). Diacylglycerol and PC (130 nmol of each) were dried under nitrogen and resuspended in 25 µl of 200 mM MOPS (pH 7.5), 80 mM $MgCl_2$, 26 mM Triton X-100 and 25 µl of dH_2O. This mixture was sonicated for 5 minutes until translucent and then incubated at 37°C for 5 minutes. Microsomes (25-100 µg) and dH_2O were added to 50µl of the sonicated assay mix to a final volume of 90 µl, and the mixture was incubated at

room temperature for 5 minutes to allow micelles to form. The reaction was initiated by the addition of 10 µl of 5 mM [^{14}C]CDP-choline (1000 cpm/nmol) and incubated at 25°C for 30 minutes. The reaction was terminated with 3 ml of $CHCl_3/CH_3OH$ (2/1) and 1.4 ml of 0.9% KCl, vortexed well, and centrifuged at 2500 rpm for 10 minutes. The upper aqueous phase was aspirated off and the lower organic phase was washed twice with 2 ml of 40% CH_3OH. A sample of the organic phase was dried in a scintillation vial and radioactivity was determined.

v) Phospholipase C

Diacylglycerols not commercially available were prepared from PC by digestion with *Bacillus cereus* phospholipase C. PC was dried under nitrogen in a screw-cap test tube, resuspended in 5 ml of water-washed diethylether, and re-dried under nitrogen. The PC was resuspended in 5 ml of water-washed diethylether and 2 ml of 50 mM Tris-HCl (pH7.5) was added. Next, 10 µl of *B. cereus* phospholipase C (1000U/0.22 ml; Sigma) was added, the mixture was vortexed, and the cap was screwed on. The tube was placed on a rotator and incubated at room temperature overnight. The top (ether) phase was transferred to a new test tube, dried under nitrogen, and resuspended in 1.5 ml of $CHCl_3/CH_3OH$ (2/1) and 0.75 ml of H_2O. The mixture was vortexed and centrifuged at 2500 rpm for 10 minutes to separate phases. The upper phase was aspirated off and the lower phase was transferred to a new test tube and dried under nitrogen. A sample was subjected to TLC in a solvent system of heptane/diethylether/acetic acid (25/75/1). R_f values are as follows: PC, 0; 1,2-DAG, 0.3, 1,3-DAG, 0.4. Diacylglycerol yield was estimated using an ester assay (Stern and Shapiro, 1953).

vi) DAG kinase

Diacylglycerol levels were measured using a DAG kinase assay (Preiss et al., 1986). Lipid extract was added to a 10-ml glass tube and solvent was evaporated under nitrogen. Twenty microliters of 7.5% octylglucoside and 5 mM cardiolipin was added and the mixture was vortexed and sonicated at 25°C for 15 seconds. After incubating at 25°C for 10 minutes, 50 µl of 2X reaction buffer (100 mM imidazole-HCl [pH 6.6], 100 mM NaCl, 25 mM $MgCl_2$, 2 mM EDTA), 10 µl of 20 mM DTT, and 10 µl of DAG kinase (InterSciences; 1 mg/ml stock diluted 1:1 with 10 mM imidazole [pH 6.6] prior to use) was added. The mixture was vortexed, 10 µl of 10 mM [γ-^{32}P]ATP was added, and the reaction was incubated at 25°C for 45 minutes. The reaction was terminated by the addition of 3 ml of $CHCl_3/CH_3OH$ (1/2, v/v), followed by 0.7 ml of 1% (v/v) $HClO_4$. After vortexing, 1 ml each of $CHCl_3$ and 1% $HClO_4$ was added, and the mixture was re-vortexed and centrifuged at 1500 rpm for 5 minutes to separate phases. The lower phase was washed twice with 2 ml of dH_2O and extracts were dried under nitrogen. One-half to one-third of the product was applied to a TLC plate, which was developed in $CHCl_3/CH_3OH$/acetic acid (65/15/5, v/v). The phosphorylated products were visualized by exposing the TLC plate to x-ray film for 2-4 hours at -70°C with an intensifying screen. Spots were scraped into scintillation vials and the radioactivity was determined by scintillation counting.

IV. Results

A. Human choline/ethanolaminephosphotransferase 1 (hCEPT1)

1. Identification of hCEPT1 and analysis of hCEPT1p

A TBLASTN search (Gish and States, 1993) was performed against the Expressed Sequence Tag (EST) database, with the use of default parameters, with reference to the predicted amino acid sequences of both the entire *S. cerevisiae* Cpt1p coding region and the Cpt1p CDP-alcohol phosphotransferase motif. Several ESTs were identified that possessed similarity to either the Cpt1p CDP-alcohol phosphotransferase motif or to other regions within the Cpt1p protein. These ESTs were obtained and sequenced. One cDNA (human Jurkat T-cell, Genbank™ accession number AA312638) contained a full-length open reading frame with a high degree of similarity to the yeast Cpt1p, including a diagnostic CDP-alcohol phosphotransferase motif region. This 2051-base pair cDNA was sequenced in its entirety on both strands and submitted to Genbank™ (accession number AF068302) and the complete nucleotide sequence is shown in Figure 6. An open reading frame of 1248 base pairs was identified within the EST and corresponds to a protein of 416 amino acids with a predicted molecular weight of 46,550 Da (Figure 7: Genbank™ accession number AAD25170). hCEPT1p is composed of 48.6% hydrophobic residues, 39.4% uncharged polar residues, and 12% charged residues with a net charge of +6 at neutral pH. Inspection of the primary amino acid sequence revealed that the CDP-alcohol phosphotransferase motif, $DG(X)_2AR(X)_8G(X)_3D(X)_3D$, spans residues 136-158 of hCEPT1p. At the onset of this work, only three choline/ethanolaminephosphotransferases were known, Cpt1p and Ept1p from *S. cerevisiae*, and CDP-aminoalcoholphosphotransferase (AAPT1p) from *Glycine max* (soybean). An alignment

GGCACGAGCT GGAGTCGGAG GCGATATTTC TAGGGGTGTA CTTGTTGGGG TCAGGGTAAG CACCAGCCAC
AAAAACCTAC AAAAGAAGGG AAATTACTGT CTTTAAATAT TAAAAAAAAA CAAGATCCAT GAGTGGGCAT
CGATCAACAA GGAAAAGATG TGGAGATTCT CACCCGGAGT CCCCAGTGGG CTTCGGGCAT ATGAGTACTA
CAGGATGTGT ATTAAATAAA TTGTTTCAGT TACCAACACC ACCATTGTCA AGACACCAAC TAAAGCGGCT
AGAAGAACAC AGATATCAAA GTGCTGGACG GTCCCTGCTT GAGCCCTTAA TGCAAGGGTA TTGGGAATGG
CTCGTTAGAA GAGTTCCCTC CTGGATTGCC CCAAATCTCA TCACCATCAT TGGACTGTCA ATAAACATCT
GTACAACTAT TTTATTAGTC TTCTACTGCC CTACAGCTAC AGAGCAGGCA CCTCTGTGGG CATATATTGC
TTGTGCCTGT GGCCTTTTCA TTTACCAGTC TTTGGATGCT ATTGATGGGA AACAGGCAAG AAGAACCAAT
AGTAGTTCTC CTCTGGGAGA ACTTTTTGAT CATGGCTGTG ATTCACTATC AACAGTTTTT GTGGTTCTTG
GAACTTGTAT TGCAGTGCAG CTGGGGACAA ACCCTGATTG GATGTTTTTT TGTTGTTTTG CGGGGACATT
TATGTTCTAT TGTGCGCACT GGCAAACGTA TGTTTCTGGA ACATTGCGAT TTGGAATAAT TGATGTGACT
GAAGTGCAAA TCTTCATAAT AATCATGCAT TTGCTGGCAG TGATTGGAGG ACCACCTTTT TGGCAATCTA
TGATTCCAGT GCTGAATATT CAAATGAAAA TTTTTCCTGC ACTTTGTACT GTAGCAGGGA CCATATTTTC
CTGTACAAAT TACTTCCGTG TAATCTTCAC AGGTGGTGTT GGCAAAAATG GATCAACAAT AGCAGGAACA
AGTGTCCTTT CTCCTTTTCT CCATATTGGA TCAGTGATTA CATTAGCTGC AATGATCTAC AAGAAATCTG
CAGTTCAGCT TTTTGAAAAG CATCCCTGTC TTTATATACT GACATTTGGT TTTGTGTCTG CTAAAATCAC
TAATAAGCTT GTGGTTGCAC ACATGACGAA AAGTGAAATG CATTTGCATG ACACAGCATT CATAGGTCCG
GCACTTTTGT TTCTGGACCA GTATTTTAAC AGCTTTATTG ATGAATATAT TGTACTTTGG ATTGCCCTGG
TTTTCTCTTT CTTTGATTTG ATCCGCTACT GTGTCAGTGT TTGCAATCAG ATTGCGTCTC ACCTGCACAT
ACATGTCTTC AGAATCAAGG TCTCTACAGC TCATTCTAAT CATCATTAAT GATGTAATTG GTATATAGGA
ACATCATGTT TTCTGCAGGA AAGAAAGTAA CATATTAAGG AGAATGGGGG TGGATAAGAA CAAATATAAT
TTATAATAAT CAATGTTGTA TAACTTTTAT TCTTTATTAT TGGTAACACG CCCTAACTAT CCTGTGTGAG
AATGGGAATT TCAAGTCCCA TCTTGTAAAT TGTATATGTT GTCATGCAGG GTTTGGGCCA AGAAAGCATG
CAGAAAAAAA TGCCATGTGA TTGTAATTAT CCTGGATTCA GAATAATACT GTGATGGGGA GCCAGATCCG
CAGTGGTGGA GAGTTCTAAT GTTGACTGTT TGCAGGCCAA AAGATGATTG CTTTATAATT TTAACAAATC
ATTGTCTTTT AGTAACATCC TTGTTTAGTG TCTTCTCAAG CTTTCTTTAC TGAGGAATTC AGCTTGTGAC
ACAGATACAT CCCACTAGCT TGTGAGGTGG AACTAGTAAT AAAGACCTTG AATTTGGATT GAAAAGTTTC
CTATCTTTAC ATTGTTGAGG AAGTCCTTTT TTTTTTTTTT TTTAATTGCT CAAGAAATGA TTCTCTCACA
GGCTTGGGAA ATCCTGTTAG CATGCAGAAT AATGTGGTAA CTTTGTCAAT TTCCCATTTT ATTTTTTTAA
ATAAATATAT GATCTAAACG G

Figure 6. Nucleotide sequence of AF068302 (hCEPT1). The 2051-base pair EST was sequenced in its entirety on both strands. An open reading frame of 1248 base pairs corresponding to hCEPT1 is underlined.

```
1 M S G H R S T R K R C G D S H P E S P V G F G H
M S T T G C V L N K L F Q L P T P P L S R H Q L K
R L E E H R Y Q S A G R S L L E P L M Q G Y W E W
L V R R V P S W I A P N L I T I I G L S I N I C T
T I L L V F Y C P T A T E Q A P L W A Y I A C A C
G L F I Y Q S L D A I D G K Q A R R T N S S S P L
G E L F D H G C D S L S T V F V V L G T C I A V Q
L G T N P D W M F F C C F A G T F M F Y C A H W Q
T Y V S G T L R F G I I D V T E V Q I F I I I M H
L L A V I G G P P F W Q S M I P V L N I Q M K I F
P A L C T V A G T I F S C T N Y F R V I F T G G V
G K N G S T I A G T S V L S P F L H I G S V I T L
A A M I Y K K S A V Q L F E K H P C L Y I L T F G
F V S A K I T N K L V V A H M T K S E M H L H D T
A F I G P A L L F L D Q Y F N S F I D E Y I V L W
I A L V F S F F D L I R Y C V S V C N Q I A S H L
H I H V F R I K V S T A H S N H H - 416
```

Figure 7. Predicted amino acid sequence of hCEPT1p. The CDP-alcohol phosphotransferase motif is underlined.

of hCEPT1p with Cpt1p, Ept1p, and AAPT1p is shown in Figure 8. Overall identities and similarities as compared with hCEPT1p were: 23.1% and 38.2% for yeast Cpt1p; 21.9% and 34.6% for yeast Ept1p; and 26.9% and 41.8% for AAPT1p. A search of the human genome database revealed that the *CEPT1* gene is located on chromosome 1p36.13-q23.3. The gene is 44.7 kb in length and is comprised of 8 exons.

To construct a model of the hCEPT1 protein, the primary amino acid sequence was analyzed by several methods of structural prediction. First, the probable membrane-spanning helical regions were identified by the method of Kyte and Doolittle (Kyte and Doolittle, 1982) As shown in Figure 9A, at least seven regions of sufficient length and hydrophobicity to span the membrane were revealed. This finding is consistent with the structural predictions for Cpt1p and Ept1p from *S. cerevisiae* (Hjelmstad and Bell, 1990; Hjelmstad and Bell, 1991b). Interestingly, a predicted α-helix from residues 151-168 demonstrates an amphipathic character with a highly charged face on one side of the helix and a hydrophobic face on the opposite side, suggesting a mode of membrane interaction (Figure 9B). In addition, the final two aspartate residues of the CDP-alcohol phosphotransferase motif, the known catalytic residues within the Cpt1p enzyme, reside within this amphipathic helix. Similar amphipathic helices are predicted in Cpt1p, Ept1p, and AAPT1p. We suggest that the amphilicity of this helix is required to allow the interfacing of the hydrophilic CDP-alcohol substrate with the hydrophobic diradylglycerol substrate. Membrane-spanning domains were also predicted for hCEPT1p by using the TMpred algorithm and positioned within the sequence. Depending on the algorithm, used, the primary sequence of hCEPT1 predicts between 6-8 membrane-spanning domains. Based on previous data regarding the structure of Cpt1p and Ept1p

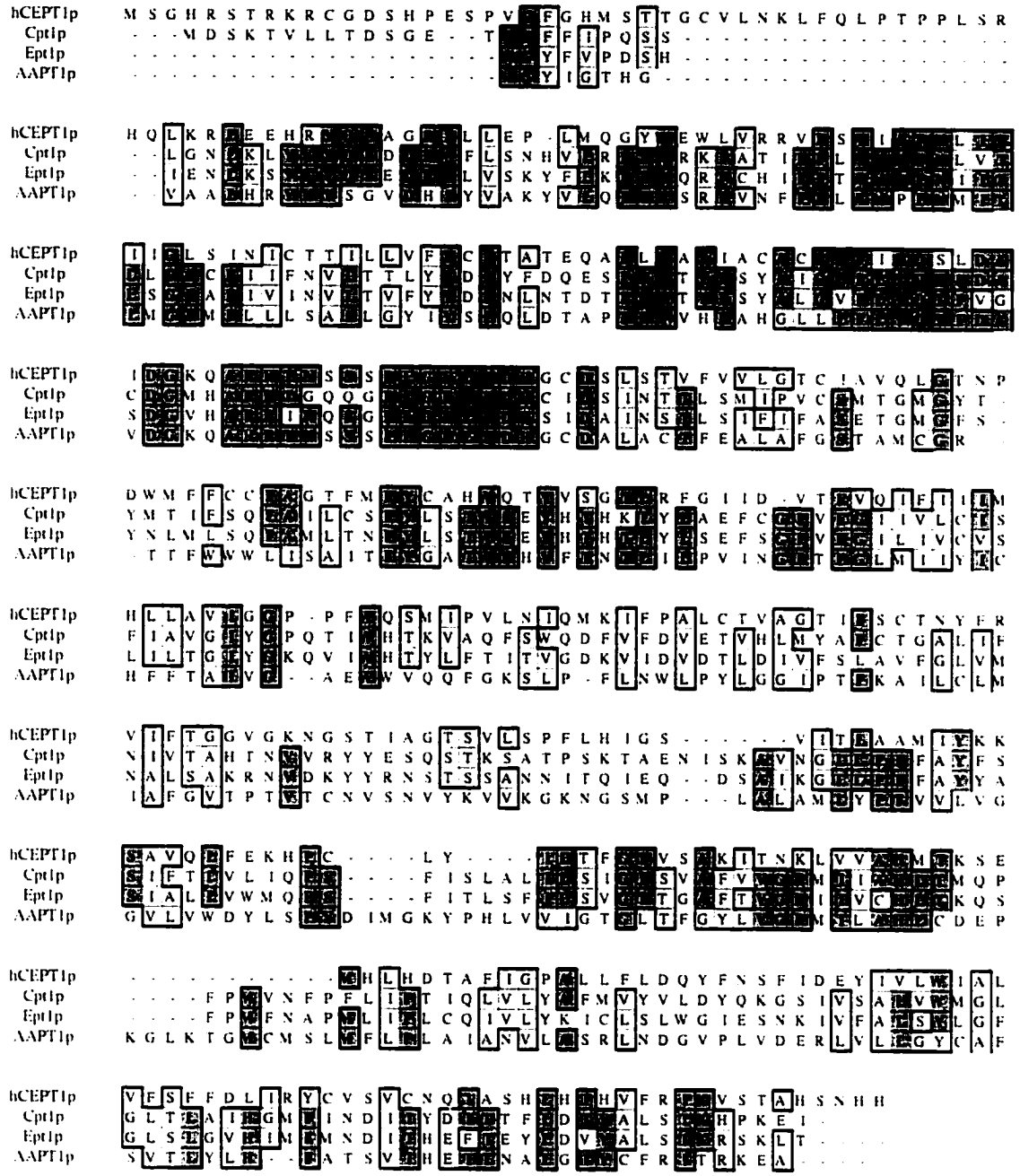


Figure 8. Multiple alignment of known choline/ethanolaminephosphotransferases. The amino acid sequences of human choline/ethanolaminephosphotransferase (hCEPT1p), yeast cholinephosphotransferase (Cpt1p), yeast ethanolaminephosphotransferase (Ept1p), and soybean aminoalcoholphosphotransferase (AAPT1p) were aligned using CLUSTALW.

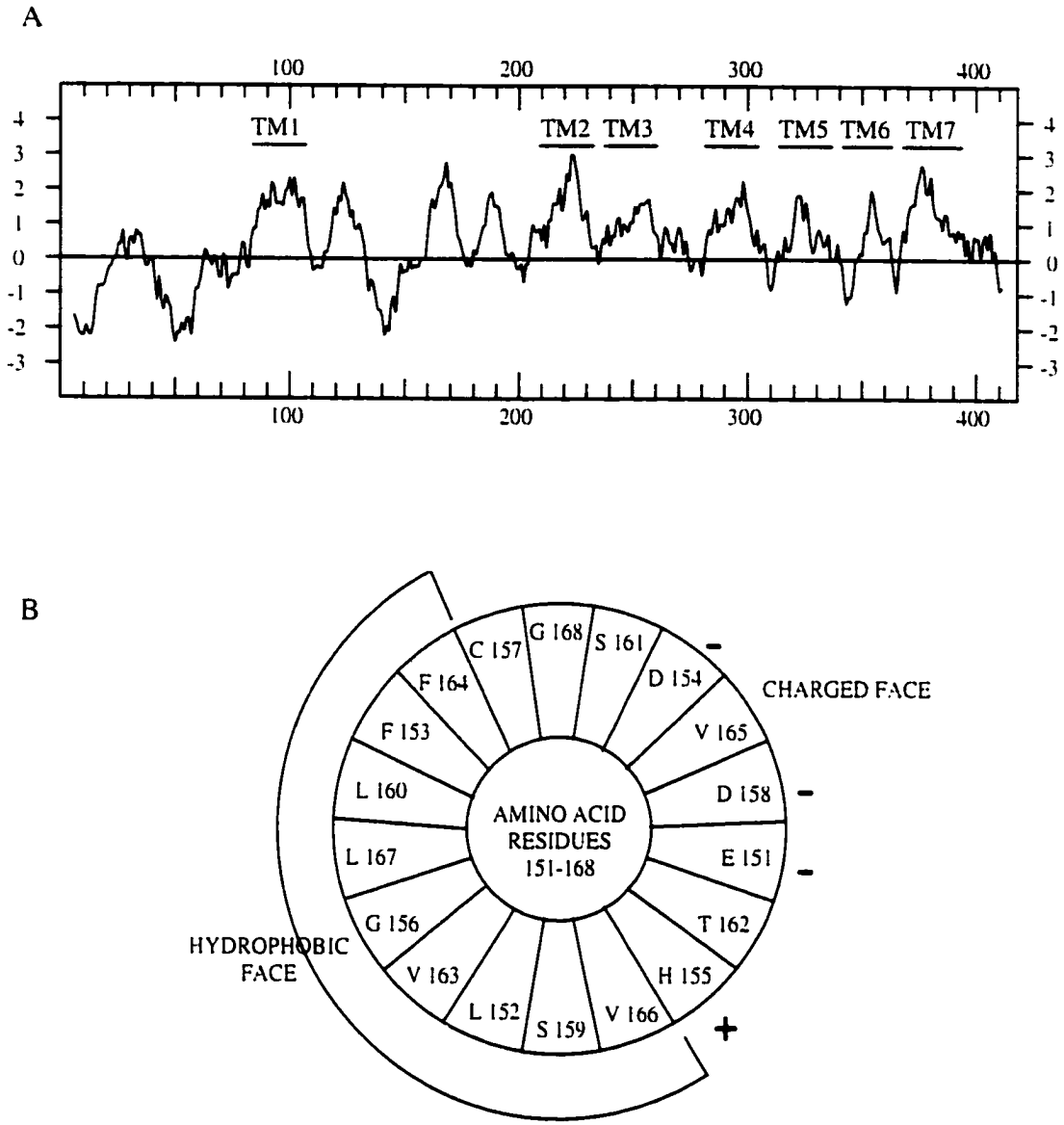


Figure 9. Predicted secondary structure of hCEPT1p. (A) Kyte-Doolittle hydropathy plot for hCEPT1p. The degree of hydrophobicity for each amino acid is indicated on the x-axis. TM. transmembrane domain. (B) The positioning of amino acid residues of hCEPT1p in a predicted amphipathic helix within the catalytic domain.

from *S. cerevisiae*, and the identity of the hCEPT1 sequence with the yeast sequences, we predict that hCEPT1p contains seven membrane-spanning helices as shown in Figure 10. The first three membrane-spanning helices are predicted to bind diacylglycerol, while the large cytoplasmic loop between residues 107 and 180 is predicted to bind the CDP-aminoalcohol substrate.

2. Expression and enzymatic characterization of hCEPT1p

An expression system devoid of endogenous CPT and EPT activities was sought for expression and subsequent enzymological analysis of hCEPT1p. Two systems were explored: (1) prokaryotes are devoid of Kennedy pathways, so hCEPT1 was expressed in *E. coli*, and (2) hCEPT1 was expressed in *S. cerevisiae* strain HJ091 (*cpt1::LEU2 ept1*) which contains null mutations at the loci coding for its endogenous cholinephosphotransferase (*CPT1*) and choline/ethanolaminephosphotransferase (*EPT1*). The open reading frame of the hCEPT1 cDNA was subcloned into pET23a (pAH5) and p416 GPD (pAH9) such that a T7-epitope tag was added to the N-terminus of the protein for subsequent immunological detection. Currently, there are no antibodies to detect hCEPT1p. Western blot analysis of T7-epitope-tagged hCEPT1p revealed that a band of approximately the expected molecular mass, 46.5 kDa, was produced in both *S. cerevisiae* and *E. coli* (Figure 11). All of the hCEPT1p detected in *S. cerevisiae* was full length, with little to no degradation. However, most of the hCEPT1p synthesized in *E. coli* had undergone proteolytic degradation, consistent with the fact that *E. coli* do not possess PC or the enzymes of the Kennedy pathway. Therefore, the bacteria probably do not have the necessary machinery for proper folding of the protein within the membrane. Based on these results, heterologous expression of hCEPT1p in *S. cerevisiae* HJ091 was

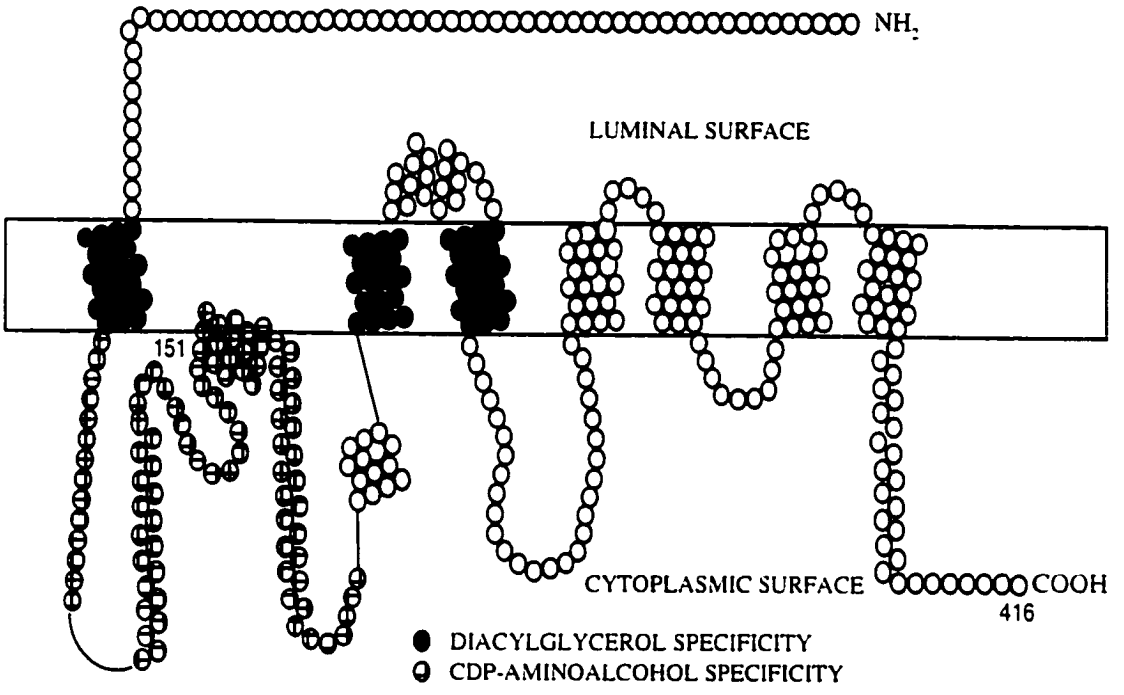


Figure 10. Proposed membrane topography of hCEPT1p. Each circle represents an amino acid in the hCEPT1 protein.

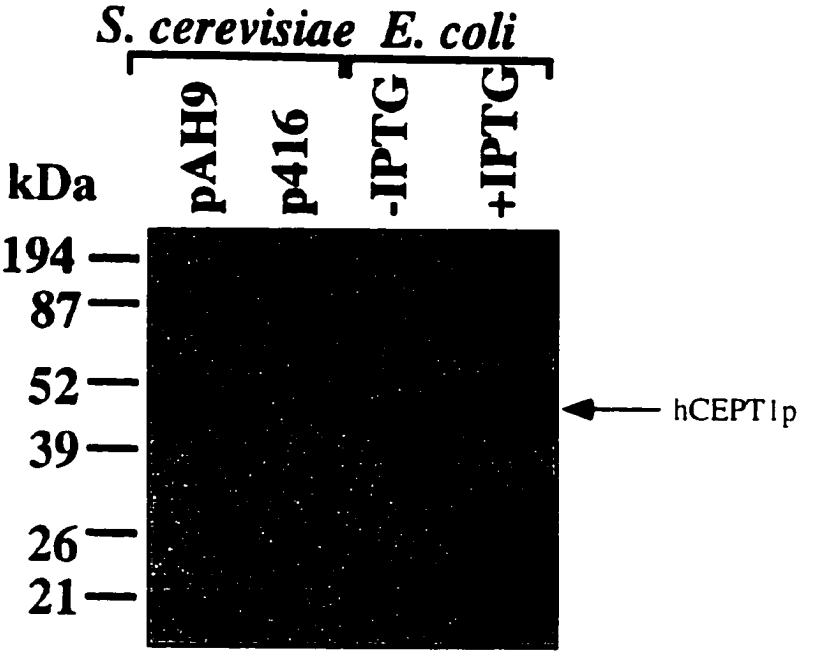


Figure 11. Western blot of hCEPT1p expressed in *S. cerevisiae* or *E. coli*. Membrane preparations from *S. cerevisiae* strain HJ091 (*cpt1::LEU2 ept1*) containing pAH9 or p416 GPD. or from *E. coli* BL21 (DE3) *pLysS* containing pAH5 (with or without induction of protein with 0.4 mM IPTG for 2 hours at 25°C) were separated by 12% SDS-PAGE and blotted with T7-HRP (1:5000) as described in Materials and Methods. The positions of molecular mass markers are indicated at the left.

utilized for subsequent analyses.

Initial enzymatic characterization of hCEPT1p suggested that it was capable of utilizing both CDP-choline and CDP-ethanolamine as substrates *in vitro*. Therefore, subsequent enzymatic analyses were directed towards the study of both the CPT and EPT reactions. Previous studies, using both crude and partially purified membrane preparations *in vitro* as well as metabolic labelling experiments *in vivo*, have described that CPT activity prefers saturated and monounsaturated diacylglycerols as substrates. In contrast, EPT prefers diacylglycerols with polyunsaturated fatty acids at the *sn*-2 position, with a particular preference for hexaenoic (22:6) species (Samborski et al., 1990). *In vitro* enzyme activities were determined via a non-mixed micelle assay in microsomal membranes from HJ091 expressing hCEPT1 from pAH9. As shown in Table 3, obvious differences in the capability of hCEPT1p to catalyze phosphobase transfer from either CDP-choline or CDP-ethanolamine to various DAGs were apparent. Of the DAGs tested, CDP-choline preferred di10:0 >> di16:1 > di8:0 = di18:1 = 16:0/22:6, whereas CDP-ethanolamine preferred di18:1 > di16:1 = 16:0/18:1 = 16:0/22:6. A kinetic analysis of hCEPT1p with di18:1 as the DAG substrate in the presence of either CDP-choline or CDP-ethanolamine revealed a K_M (app) of 37 μM and a V_{max} (app) of 10.5 nmol min^{-1} mg^{-1} for CDP-choline, and a K_M (app) of 101 μM and a V_{max} (app) of 4.35 nmol min^{-1} mg^{-1} for CDP-ethanolamine (Figure 12A). Similar results were obtained with $C_{16:0}/C_{22:6}$ DAG as a substrate, with a K_M (app) of 28 μM and a V_{max} (app) of 10.1 nmol min^{-1} mg^{-1} for CDP-choline, and a K_M (app) of 133 μM and a V_{max} (app) of 2.41 nmol min^{-1} mg^{-1} for CDP-ethanolamine (Figure 12B).

Several investigations attempting to purify mammalian CPTs or EPTs have resulted

Table 3. *In vitro* enzymatic activity of hCEPT1p

	Enzyme activity (nmol min^{-1} mg^{-1})	
Diacylglycerol	**CPT**	**EPT**
di8:0	11.0 ± 1.3	ND[1]
di10:0	34.5 ± 2.0	ND
di12:0	5.5 ± 1.4	ND
di14:0	1.4 ± 0.05	ND
di16:0	0.9 ± 0.03	ND
di16:1	14.2 ± 1.7	1.8 ± 0.2
di18:1	10.3 ± 0.5	4.0 ± 0.2
16:0/18:1	7.5 ± 0.4	2.0 ± 0.2
16:0/22:6	10.3 ± 0.7	1.0 ± 0.1
18:0/22:6	2.5 ± 0.1	ND

ND[1]: not detectable

Choline- and ethanolaminephosphotransferase activities were determined for microsomal preparations from *S. cerevisiae* HJ091 (*cpt1::LEU2 ept1*) expressing hCEPT1p from pAH9 using the Tween 20 (0.004%) assay as described in Materials and Methods. Results are shown as the mean ± SEM for at least four experiments.

Figure 12. Kinetic analysis of hCEPT1p. Enzyme activities were determined using the Tween 20 (0.004%) assay for microsomal membranes from *S. cerevisiae* HJ091 (*cpt1::LEU2 ept1*) cells expressing hCEPT1 from pAH9 essentially as described in Materials and Methods except for the CDP-aminoalcohol concentrations, which were as shown. Results are shown as the mean of four experiments. The substrates used were (A) di18:1 diacylglycerol and (B) 16:0/22:6 diacylglycerol.

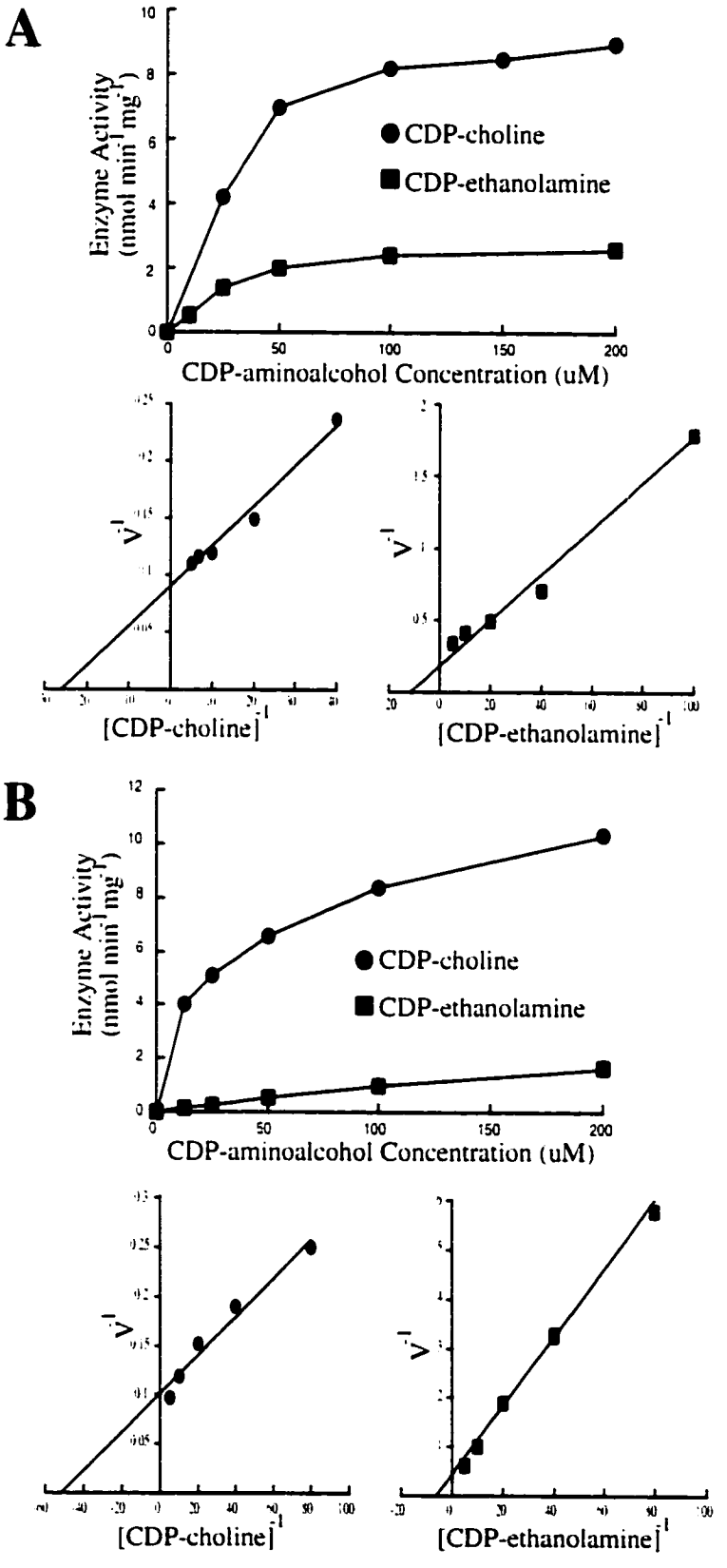

A
Enzyme Activity
(nmol min-1 mg-1)
CDP-choline
CDP-ethanolamine
CDP-aminoalcohol Concentration (uM)
v-1
[CDP-choline]-1
[CDP-ethanolamine]-1
B
Enzyme Activity
(nmol min-1 mg-1)
CDP-choline
CDP-ethanolamine
CDP-aminoalcohol Concentration (uM)
v-1
[CDP-choline]-1
[CDP-ethanolamine]-1

Figure 12

in the resolution of separate peaks of activities with various CDP-aminoalcohol specificities that differed in their ability to use Mg^{2+} or Mn^{2+} as the essential cation (Kanoh and Ohno, 1976; McMaster and Bell, 1997; O et al., 1989). The ability of hCEPT1p to utilize both CDP-choline and CDP-ethanolamine as substrates prompted the examination of its ability to use Mg^{2+} or Mn^{2+} as the essential cation cofactor with respect to each substrate (Figure 13). Both the CPT and EPT activities of hCEPT1 required millimolar levels of either Mg^{2+} or Mn^{2+} for activity to be detected. Mg^{2+} activated the CPT activity of hCEPT1 to a 4-fold greater extent than Mn^{2+}, whereas the EPT activity was preferentially activated by Mn^{2+} at 10 mM (2.5-fold more activity than Mg^{2+}), but was inhibited at higher Mn^{2+} concentrations, resulting in activities that approached those of Mg^{2+} activation. These are essentially identical results with those observed by Kanoh and Ohno (Kanoh and Ohno, 1976) for a partially purified choline/ethanolaminephosphotransferase activity from rat liver. The precise mechanism by which cations activate CPTs or EPTs has yet to be resolved; both cation-CDP-aminoalcohol and cation-enzyme complexes have been proposed. Work on chimeric yeast Cpt1p/Ept1p enzymes predicted that Mg^{2+} is used *in vivo* (Hjelmstad et al., 1994; McMaster et al., 1996); however, resolution awaits the availability of pure protein.

A cholinephosphotransferase has been implicated in the *de novo* synthesis of platelet-activating factor (PAF) (Snyder, 1997; Woodard et al., 1987). This putative PAF-specific CPT was identified based on DTT susceptibility of CPT activity measured in microsomal membranes, with PAF-specific activity being DTT resistant and PC-specific activity being DTT sensitive; the PAF-specific activity was slightly stimulated by DTT, whereas the PC-specific activity was greatly inhibited by DTT. Using the same assay as

Figure 13. Cation requirements of hCEPT1p. Enzyme activities were determined using the Tween 20 (0.004%) assay for microsomal membranes from *S. cerevisiae* HJ091 (*cpt1::LEU2 ept1*) cells expressing hCEPT1 from pAH9 essentially as described in Materials and Methods except for cation concentrations, which were as shown. Results are shown as the mean ± SEM for four experiments. The substrates used were (A) CDP-choline and (B) CDP-ethanolamine.

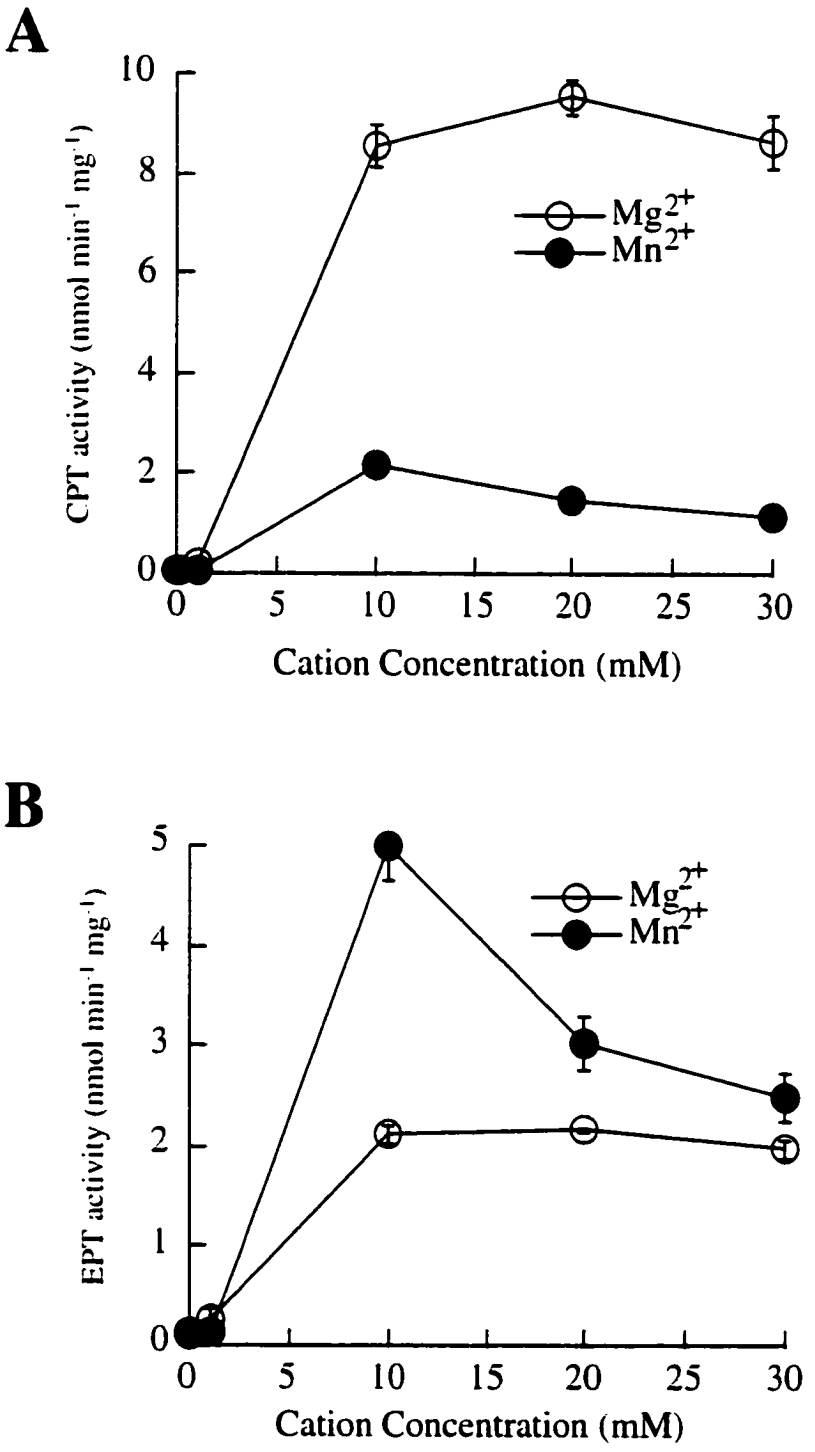

A
CPT activity (nmol min-1 mg-1)
10
8
6
4
2
0
0
5
10
15
20
25
30
Cation Concentration (mM)
Mg2+
Mn2+
B
EPT activity (nmol min-1 mg-1)
5
4
3
2
1
0
0
5
10
15
20
25
30
Cation Concentration (mM)
Mg2+
Mn2+

Figure 13

previously used in these studies (0.004% Tween 20), we tested hCEPT1p for the ability to synthesize PAF and PAF precursor *in vitro* and observed enzyme activities of 24.9 nmol min^{-1} mg^{-1} for the synthesis of PAF and 5.5 nmol min^{-1} mg^{-1} for the *de novo* synthesis of PAF precursor. However, while the hCEPT1-derived CPT activity was capable of synthesizing both PAF and PC, DTT did not alter the activity of hCEPT1 toward either substrate (Figure 14).

3. *In vivo* assessment of hCEPT1 CDP-alcohol specificity

To determine whether the *in vitro* CPT and EPT activities catalyzed by hCEPT1p could be manifested *in vivo*, radiolabelled choline or ethanolamine was added to exponential-phase HJ091 (*cpt1::LEU2 ept1*) yeast cells transformed with either p416 GPD (vector control) or pAH9 (hCEPT1 in p416 GPD). As shown in Figure 15A, the addition of radiolabelled choline to cells constitutively expressing hCEPT1 resulted in the reconstitution of the CDP-choline pathway for the synthesis of PC. Also, the expression of hCEPT1 resulted in the synthesis of PE from radiolabelled ethanolamine (Figure 15B), with the subsequent conversion of some of the radiolabelled PE to PC *via* the active PE methylation pathway present in *S. cerevisiae*. These results demonstrate that the dual specificity of hCEPT1p for CDP-choline and CDP-ethanolamine observed *in vitro* is also reflected *in vivo*. In addition, these results demonstrate that the *S. cerevisiae* and human forms of CPT and EPT are of significant functional similarity to allow the reconstitution of both the CDP-choline and CDP-ethanolamine pathways in yeast by the human enzyme.

4. Tissue distribution of hCEPT1 mRNA

A choline/ethanolaminephosphotransferase capable of synthesizing both PC and PE

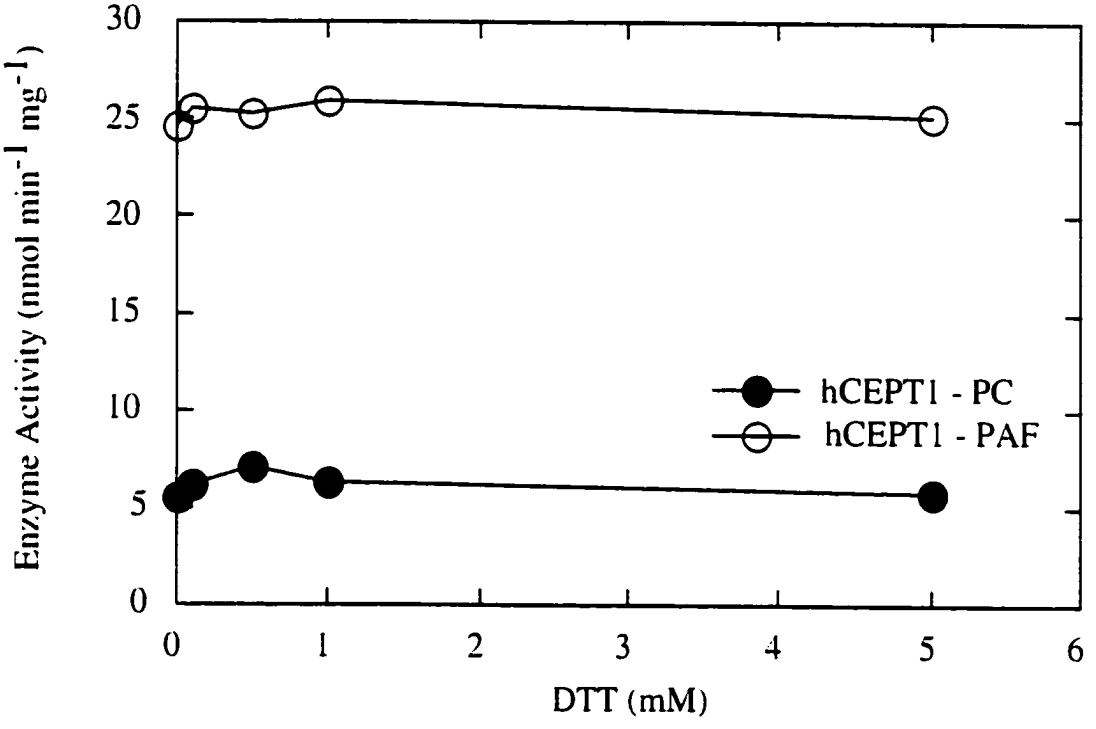


Figure 14. Effect of DTT on hCEPT1-derived synthesis of PC and PAF. Enzyme activities were determined for microsomal membranes of HJ091 yeast expressing pAH9 (hCEPT1) using the Tween 20 (0.004%) assay as described in Materials and Methods.

Figure 15. Metabolic labelling of hCEPT1 expressed in HJ091. Radiolabelled choline (A) or ethanolamine (B) was added to exponential-phase HJ091 cells expressing hCEPT1 from pAH9 for 1 hour at 30°C. The incorporation of radiolabel into phospholipids, together with the metabolites of the CDP-choline and CDP-ethanolamine pathways, was monitored and quantified as described in Materials and Methods. Results are shown as the mean ± SD for at least four separate experiments.

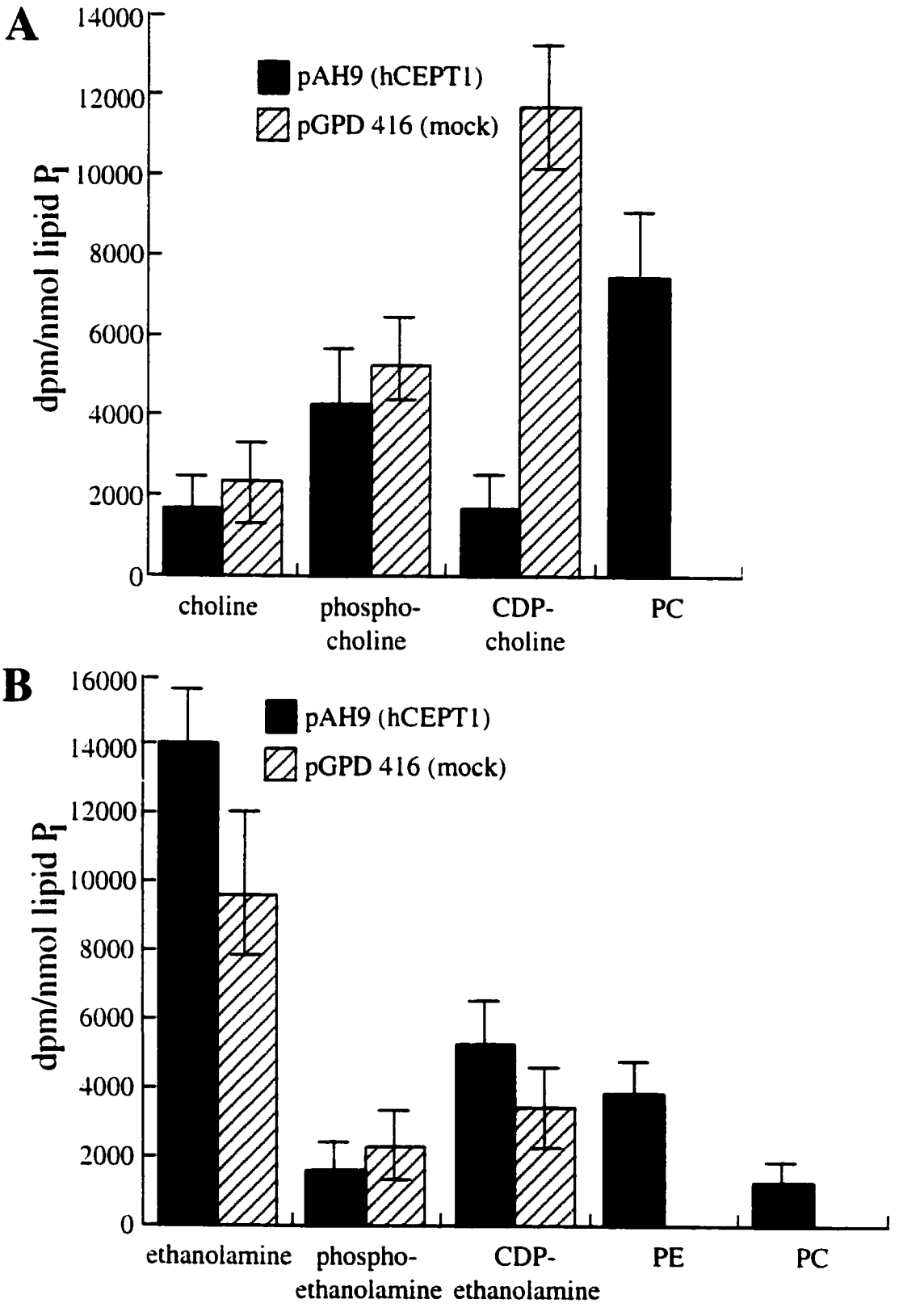

A
14000
12000
10000
8000
6000
4000
2000
0
dpm/nmol lipid Pi
pAH9 (hCEPT1)
pGPD 416 (mock)
choline
phospho-choline
CDP-choline
PC
B
16000
14000
12000
10000
8000
6000
4000
2000
0
dpm/nmol lipid Pi
pAH9 (hCEPT1)
pGPD 416 (mock)
ethanolamine
phospho-ethanolamine
CDP-ethanolamine
PE
PC

Figure 15

would be predicted to be expressed in all tissues. To assess the tissue distribution of hCEPT1, a multiple human tissue Northern blot was hybridized at high stringency with either a random-primed probe synthesized using the entire 1.2-kb hCEPT1 coding region or as a control, a 2.0-kb human β-actin cDNA. One hCEPT1 transcript of 2.3 kb was observed in all tissues examined (Figure 16). In addition, there was no obvious enrichment in any one cell type when normalized to β-actin mRNA content.

5. Subcellular localization of hCEPT1p

There have been a number of studies attempting to identity the intracellular site of *de novo* PC synthesis. As the final step in the CDP-choline pathway, the intracellular location of CPT defines the main site of PC synthesis and hence identifies the site from which *de novo* PC is transported to other organelles or is secreted for the production of lung surfactant, lipoproteins, and bile. Although CPT has most often been localized to the ER, CPT activity has also been reported in the Golgi (Vance and Vance, 1988), mitochondria (Stith and Das, 1982), and nucleus (Hunt et al., 2001), depending on the assay and subcellular fractionation method employed. We investigated the intracellular location of hCEPT1p using immunofluorescence. First, we constructed a plasmid capable of expressing an hCEPT1-GFP fusion protein by inserting a T7-tagged version of hCEPT1 into the vector pEGFP-N1, which resulted in the addition of GFP to the C-terminus of hCEPT1 (pAH20). The plasmid, pAH20, was transiently transfected into CHO-K1 cells and the cells were harvested 48 hours after transfection. To ensure that a full-length hCEPT1-GFP fusion protein was being expressed, membranes from CKO-K1 cells transiently transfected with either no DNA (mock) or various amounts of pAH20 were analyzed by Western blotting with the T7-HRP antibody (Figure 17). Transfection

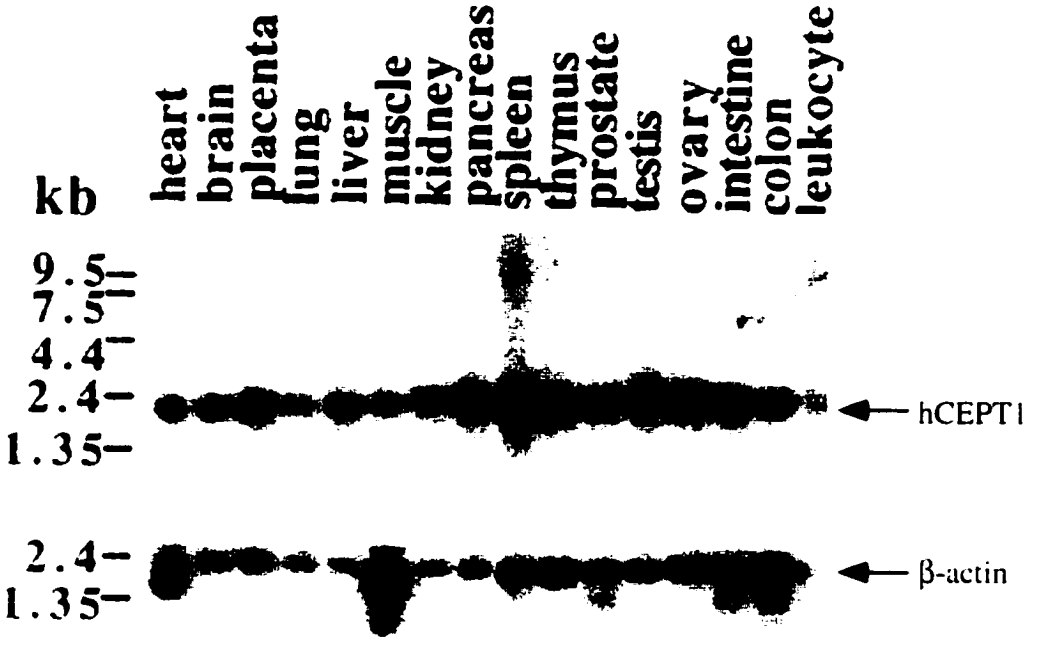


Figure 16. Northern blot analysis of hCEPT1 in human cell types. Human multiple tissue mRNA Northern blots were hybridized against probes generated using the random-priming method from the entire 1.2-kb open reading frame of hCEPT1 cDNA or a 2.0-kb human β-actin cDNA.

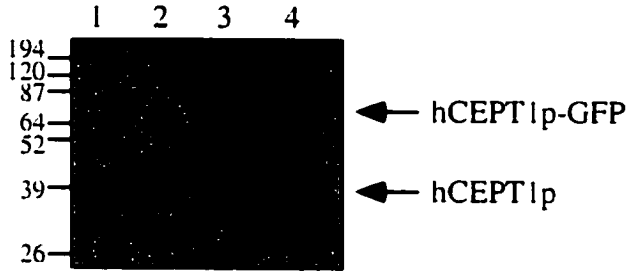


Figure 17. Expression of hCEPT1p-GFP fusion protein in CHO-K1 cells. CHO-K1 cells were transiently transfected with a vector expressing hCEPT1p with a green fluorescent protein fused to the C-terminus (pAH20). Membranes were prepared as described, and 25 µg of protein was subjected to 10% SDS-PAGE and immunoblotting with T7-HRP (1:5000). Lane 1, hCEPT1p expressed in *S. cerevisiae*; Lane 2, mock-transfected CHO-K1; Lane 3, CHO-K1 transfected with 3 µg pAH20 plasmid DNA; Lane 4, CHO-K1 transfected with 6 µg pAH20 plasmid DNA.

with both 3 μg and 6 μg of pAH20 resulted in a protein of approximately 75 kDa, which is the expected molecular mass of the fusion protein. No 75 kDa band was detected in the mock-transfected cells. Slightly more background was detected when more plasmid DNA was used; therefore subsequent transfections used 3 μg of plasmid DNA. These results demonstrate that CHO-K1 cells are capable of expressing a full-length hCEPT1-GFP fusion protein with little to no degradation.

To assess the subcellular location of hCEPT1p, we transiently transfected CKO-K1 cells with pAH20. As a positive control, CKO-K1 cells were also transiently transfected with YFP-Golgi. YFP-Golgi encodes a fusion protein consisting of enhanced yellow fluorescent protein and a sequence encoding the N-terminal 81 amino acids of human β-1,4-galactosyltransferase, which targets the fusion protein to the *trans*-medial region of the Golgi apparatus. At 48 hours post-transfection, cells were incubated with or without brefeldin A (BFA), an agent that disrupts the Golgi apparatus, and cells were fixed and mounted as described in Materials and Methods. If hCEPT1p were localized to the Golgi, addition of BFA should alter the localization of the protein, causing a more diffuse pattern of fluorescence. However, upon addition of BFA, the subcellular localization of the hCEPT1-GFP fusion protein did not change (Figure 18), suggesting that hCEPT1p is not localized to the Golgi. Addition of BFA did cause an alteration in the localization of YFP-Golgi, indicating that the BFA treatment utilized was sufficient to cause disruption of the Golgi apparatus.

To further assess the subcellular localization of hCEPT1p, we constructed CHO-K1 cell lines that were stably expressing hCEPT1 from the plasmid pMM6, which directs the expression of a T7-tagged version of hCEPT1 from the CMV promoter. Western blotting

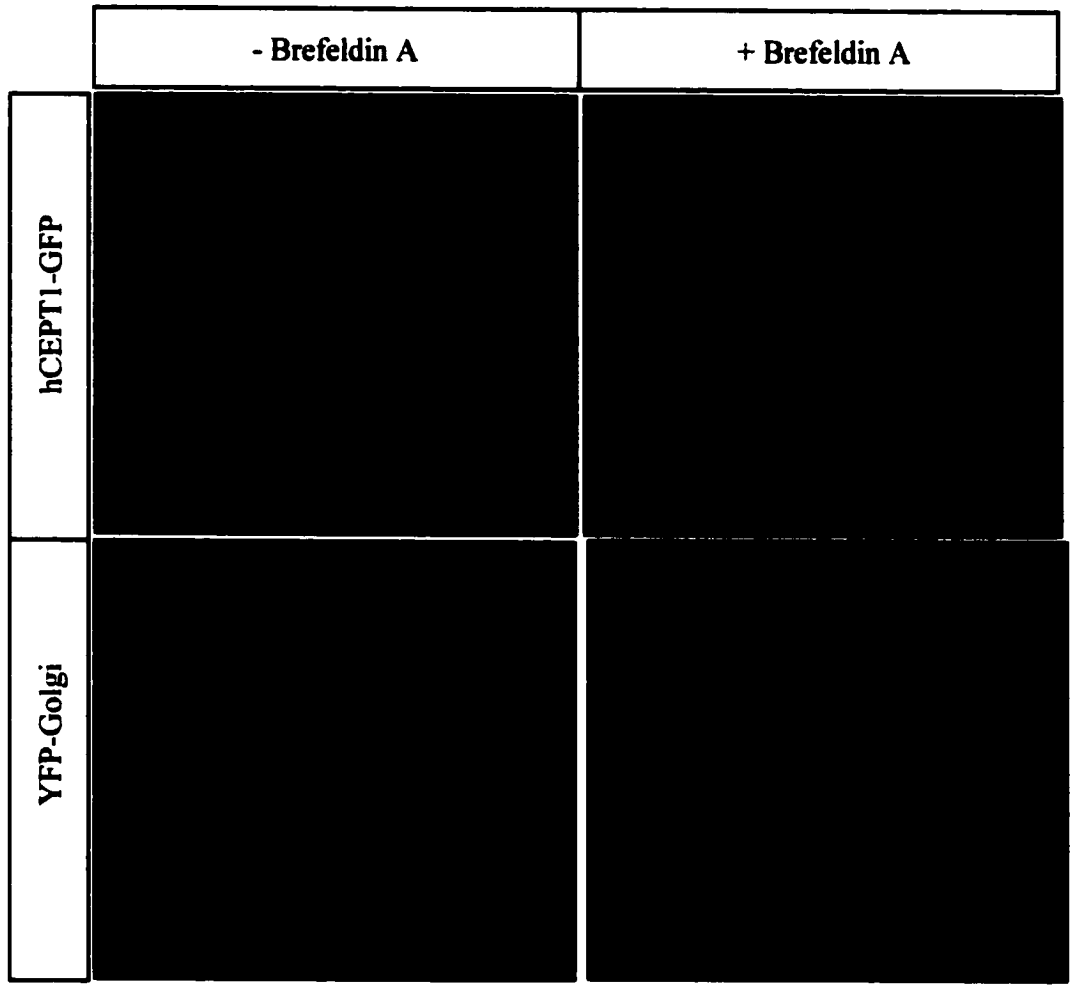


Figure 18. Effect of brefeldin A on hCEPT1p localization. CHO-K1 cells were transiently transfected with 3 μg of either pAH20 (hCEPT1-GFP) or YFP-Golgi. At 48 hours post-transfection, 2 μg/ml of brefeldin A was added for 30 minutes at 37°C to disrupt the Golgi apparatus, then cells were fixed and mounted as described in Materials and Methods.

with the T7-HRP antibody was used to identify colonies that were stably expressing pMM6. Five out of 43 clones with varying levels of expression were selected for maintenance, and are shown in Figure 19. A band of approximately 39 kDa corresponding to T7-tagged hCEPT1p was detected in the membrane fractions of each cell line. As expected, no band was detected in the cytosolic fraction. A representative of the five clones, hCEPT1-41, was used in subsequent indirect immunofluorescence studies.

Due to the lack of antibodies specific for hCEPT1p, T7-tagged hCEPT1p was detected with a monoclonal antibody to the T7-epitope tag (mT7) in CHO-K1 cells stably expressing pMM6. Cells were visualized by immunofluorescence using a goat anti-mouse secondary antibody that was conjugated to either Texas Red or FITC. In cells stably transfected with only the vector, pcDNA3, there was a very faint fluorescence in the nucleus when the T7 monoclonal antibody was used (not shown). However, when the mT7 antibody was used to detect T7-hCEPT1p in CHO-K1 cells expressing pMM6 (hCEPT-41), a fluorescent pattern suggestive of ER localization of hCEPT1p was observed (Figure 20). To verify that hCEPT1p is indeed localized to the ER, colocalization with a number a organelle-specific markers was performed. To visualize the ER, a rabbit polyclonal antibody specific for calnexin (an ER-resident protein) was utilized, followed by a secondary antibody conjugated to Texas Red. As expected, calnexin localized to reticular structures around the nucleus, characteristic of the ER (Figure 20). FITC-labelled lentil lectin was used as a marker for the Golgi apparatus and immunofluorescence revealed brightly fluorescent structures clustered at one pole of the nucleus, indicative of the Golgi (Figure 20). To visualize the mitochondria, a cell-

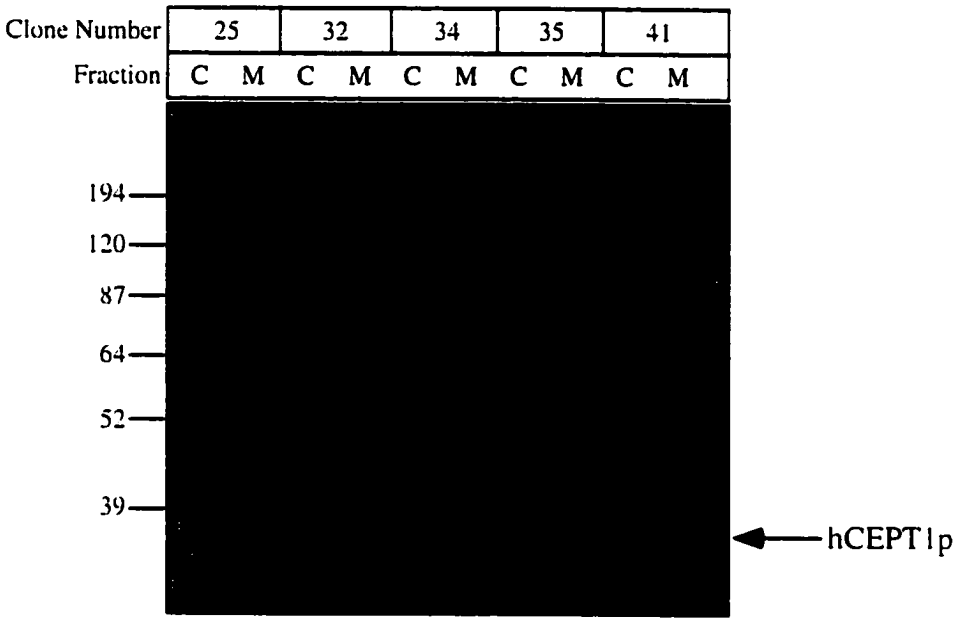


Figure 19. Western blot of CHO-K1 cells stably transfected with hCEPT1. CHO-K1 cells were stably transfected with pMM6 using the calcium phosphate method as described in Materials and Methods. Subcellular fractions were prepared from the indicated clones and 10 μg of either cytosol (C) or membrane-enriched (M) fractions were subjected to 10% SDS-PAGE and immunoblotting with T7-HRP (1:5000).

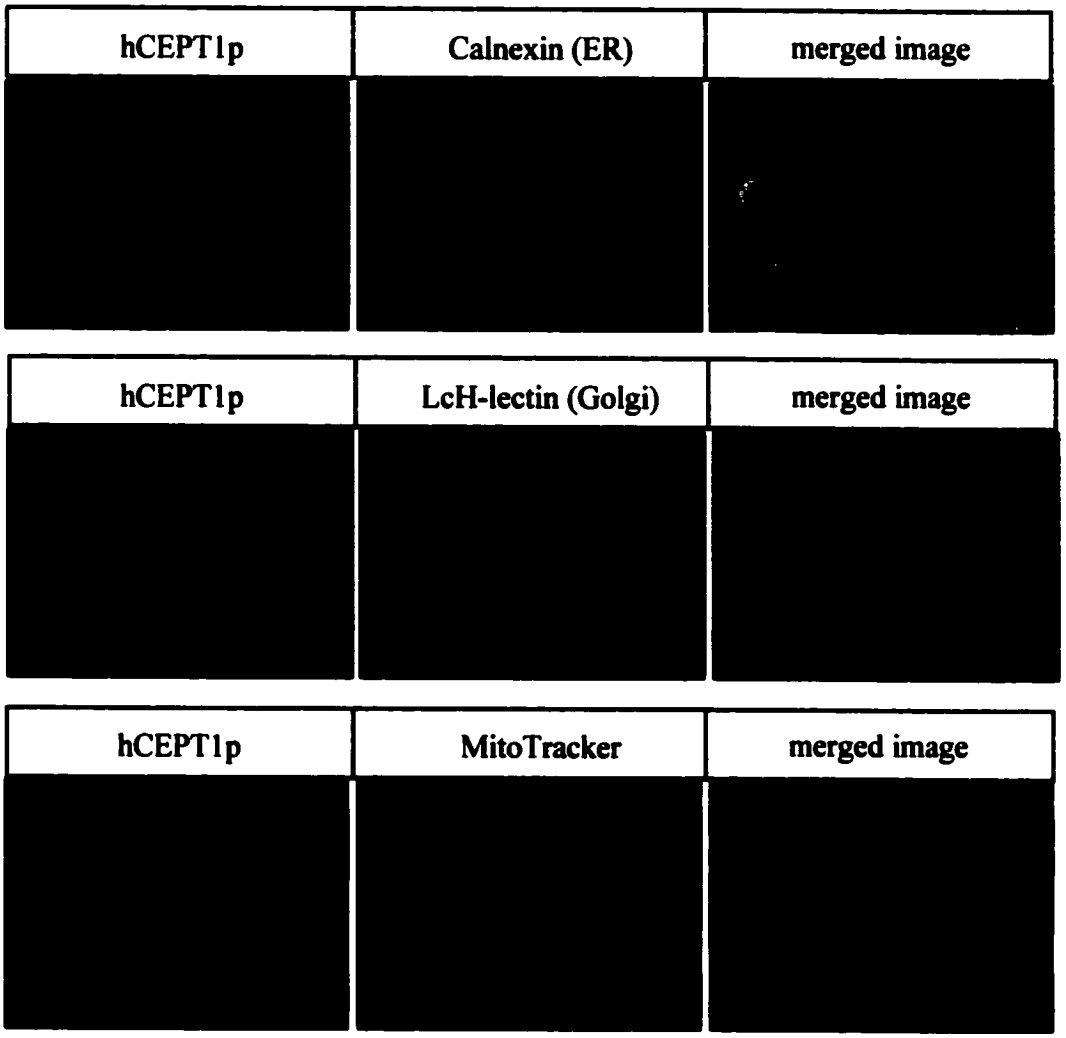


Figure 20. Subcellular localization of hCEPT1p. CHO-K1 cells stably transfected with pMM6 (T7-hCEPT1) were processed for immunofluorescence using the mT7 antibody (1:4000) to detect hCEPT1p and either α-calnexin (1:200), FITC-labelled LcH lectin (1:4000), or MitoTracker (200 nM) to detect the ER, Golgi, and mitochondria, respectively, as described in Materials and Methods. In the merged image, a yellow color indicates colocalization of the two proteins.

permeant MitoTracker® probe was used (MitoTracker® Red CMXRos), which is a mitochondrion-selective dye that is retained during cell fixation and permeabilization. Immunofluorescence revealed a large number of mitochondrial structures within the stably transfected CHO-K1 cells (Figure 20). Indirect immunofluorescence of T7-hCEPT1p together with each of the organelle-specific markers indicated that hCEPT1p is localized to the ER, and not to the Golgi apparatus or mitochondria (Figure 20). These results are consistent with the hCEPT1-GFP studies, which suggested that hCEPT1p is not localized to the Golgi apparatus (Figure 18).

In addition to the ER localization of hCEPT1p, it was noted that fluorescence also seemed to be associated with the nuclear membrane. It is also noteworthy that CTα, which catalyzes the enzymatic step immediately proceeding CPT in the *de novo* biosynthesis of PC, localizes to the nuclear membrane upon activation with oleic acid. Thus, the hypothesis was formed that CTα and hCEPT1 could colocalize at the nuclear membrane to facilitate the shuttling of CDP-choline for the synthesis of PC. To test this hypothesis, T7-hCEPT1p and CTα were detected in the hCEPT-41 cell line using mT7 and αC-CT antibodies, respectively, in the absence or presence of oleate (Figure 21). When oleate was absent, CTα was detected exclusively in the nucleus and demonstrated no colocalization with hCEPT1p. However, when CTα was activated with oleate, it translocated to the nuclear membrane and colocalized with hCEPT1p. These results suggest that in addition to its ER localization, hCEPT1p also localizes to the nuclear membrane, in close contact with CTα.

B. Substrate specificity of hCEPT1p

Characterization of hCEPT1p has demonstrated that, *in vitro*, it is capable of

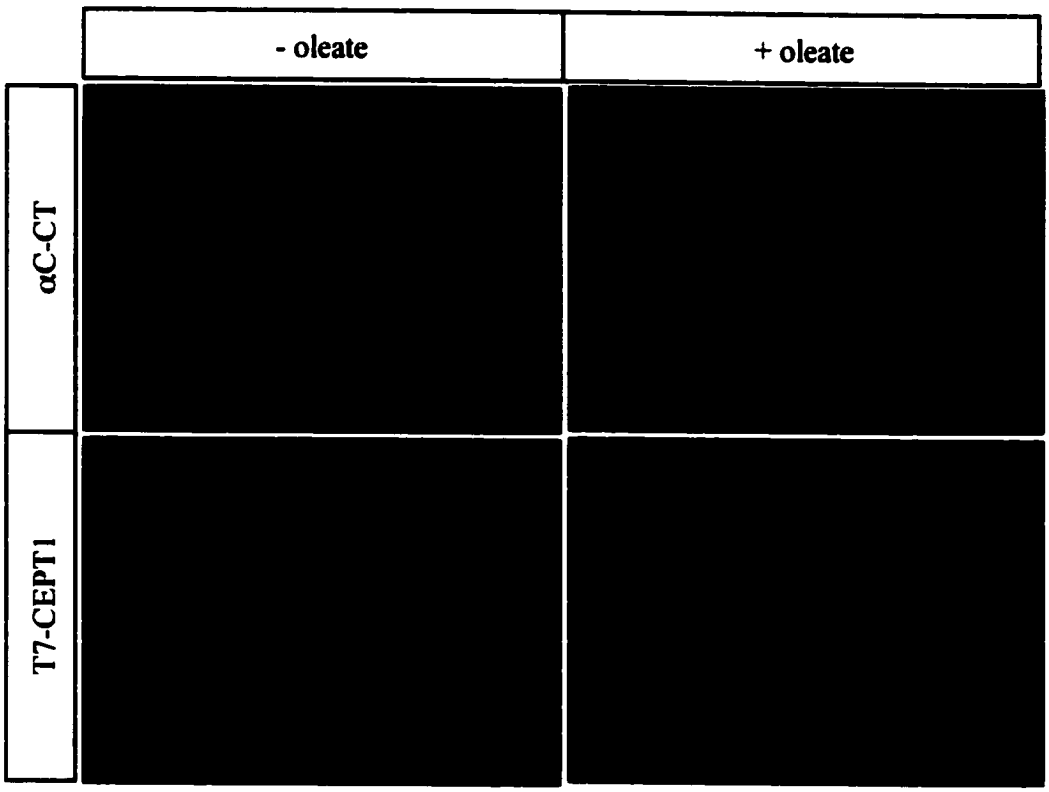


Figure 21. Colocalization of hCEPT1p and CTα in the presence of oleate. CHO-K1 cells that were stably transfected with pMM6 (T7-hCEPT1) were processed for immunofluorescence using the mT7 antibody (1:1000) to detect hCEPT1p and the αC-CT antibody (1:4000) to detect CTα as described in Materials and Methods. Oleate (500 μM in 0.5% BSA) was added for 24 hours at 37°C prior to fixing to cause translocation of CTα to the nuclear membrane.

utilizing both CDP-choline and CDP-ethanolamine as substrates in addition to a wide variety of diradylglycerol substrates (Table 3). Metabolic labelling has also shown that *in vivo*, hCEPT1p can catalyze the *de novo* synthesis of both PC and PE (Figure 15). We sought to identify residues that might be involved in CDP-aminoalcohol or DAG binding.

1. Identification of putative CDP-aminoalcohol binding residues

Chimeric enzyme studies of the *S. cerevisiae CPT1* and *EPT1* gene products identified a cytoplasmic loop region that was sufficient to confer CDP-aminoalcohol specificity (Hjelmstad et al., 1994). Within this region, a CDP-aminoalcohol phosphotransferase motif ($DG(x)_2AR(x)_8G(x)_3D(x)_3D$) was identified that is present in several phospholipid-synthesizing enzymes that catalyze the formation of a phosphoester bond utilizing a CDP-alcohol and a second alcohol as substrates (Williams and McMaster, 1998). Therefore, in an attempt to identify residues in hCEPT1p that confer CDP-aminoalcohol specificity, we aligned the phosphotransferase motif of hCEPT1p with those from *S. cerevisiae* Cpt1p and Ept1p (Figure 22A). We identified four residues that are different between hCEPT1p and Cpt1p and used site-directed mutagenesis to change the residue in hCEPT1p to the corresponding residue in Cpt1p. Based on preliminary results, two additional mutations were made that converted glycine^{156} to either serine or alanine. All mutations were constructed using the plasmid pAH9 as a template, which is a yeast expression vector directing the high-copy expression of a T7-tagged version of hCEPT1p.

2. Expression and *in vitro* enzyme activity of site-directed mutants

pAH9 plasmids carrying the various mutations were transformed into *S. cerevisiae* strain HJ091, which is devoid of endogenous CPT and EPT activity due to null mutations

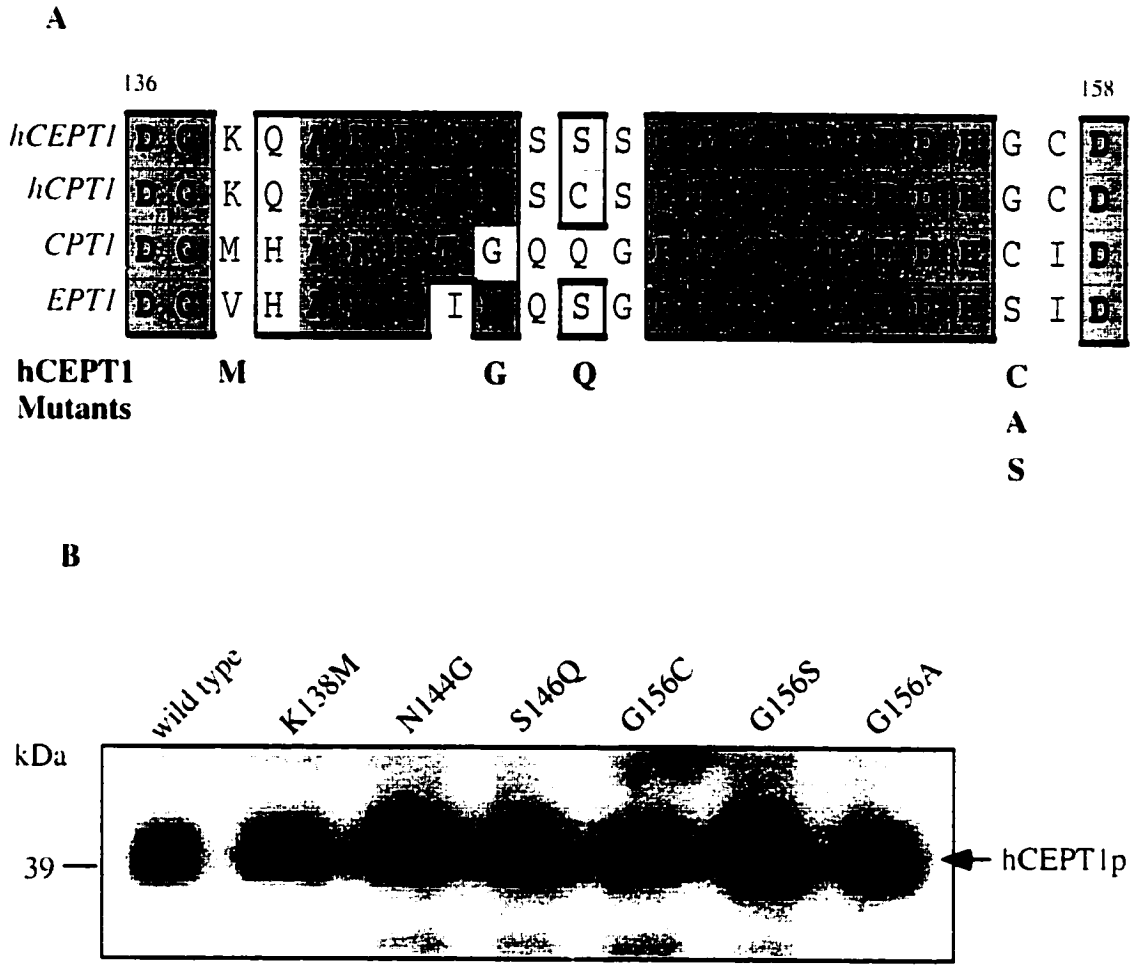


Figure 22. Identification of residues involved in CDP-alcohol substrate specificity. (A) Multiple alignment of CDP-alcohol phosphotransferase motifs from several phosphotransferases. Site-directed mutants of hCEPT1 used in this work are indicated in bold below. Dark shading indicates indentical residues and light shading indicates conserved residues. (B) Western blot of T7-tagged hCEPT1 site-directed mutants expressed in *S. cerevisiae* strain HJ091 (*cpt1::LEU2 ept1*) using the T7-HRP antibody (1:5000).

at the *CPT1* and *EPT1* loci (*cpt1::LEU2 ept1*). This ensured that any CPT or EPT activity detected would be plasmid-encoded. Western blot analysis of HJ091 membrane fractions established that similar amounts of a full-length hCEPT1 protein were being expressed in each case (Figure 22B).

The enzymatic activities of hCEPT1 mutants were determined *in vitro* using a mixed micelle assay modified from a previously published assay which used sodium cholate to deliver the DAG substrate (Bru et al., 1993). The mixed micelle assay was utilized to ensure uniformity in the delivery of the DAG substrate. As shown in Table 4, there were no significant differences observed with either the N144G or the S146Q mutations, but a decrease in both CPT and EPT activity was seen when lysine^{138} was mutated to methionine. However, this merely demonstrates an overall effect on activity, as both enzymatic activities were affected. Interestingly, mutation of glycine^{156} to cysteine abolished the ability of hCEPT1 to utilize CDP-ethanolamine as a substrate, yet retained the ability to utilize CDP-choline. Subsequent mutations of glycine^{156} to either serine (which is present in Ept1p) or alanine gave similar results to the G156C mutation, suggesting a role for glycine^{156} in CDP-ethanolamine binding.

3. *In vivo* activity of site-directed mutants

To confirm the *in vitro* results, pAH9 plasmids carrying the mutations were expressed in *S. cerevisiae* HJ091 (*cpt1::LEU2 ept1*). The ability of each mutant to reconstitute the CDP-choline pathway for the synthesis of PC and the CDP-ethanolamine pathway for the synthesis of PE was tested *via* the addition of radiolabelled choline or ethanolamine, respectively. All of the yeast expressing mutant hCEPT1 proteins were able to take up choline and synthesize PC at levels that were not significantly different

Table 4. *In vitro* enzymatic activity of CDP-alcohol mutants

	Enzyme Activity (nmol min^{-1} mg^{-1})	
Mutant	**CPT**	**EPT**
wild type	5.54	4.16
K138M	2.15	1.84
N144G	4.12	3.95
S146Q	6.20	3.02
G156C	2.36	0.20
G156S	3.11	0.34
G156A	2.20	0.21

Choline- and ethanolaminephosphotransferase activities were determined in microsomal preparations from *S. cerevisiae* HJ091 (*cpt1::LEU2 ept1*) expressing hCEPT1 CDP-aminoalcohol specificity mutants from pAH9 using the sodium cholate mixed micelle assay as described in Materials and Methods. Results are shown as the average of at least four experiments.

from yeast expressing wild-type hCEPT1p (Figure 23A). All of the yeast expressing mutant hCEPT1 proteins were also capable of taking up ethanolamine at levels similar to yeast expressing wild-type hCEPT1p. However, no hCEPT1 protein containing a substitution for glycine^{156} was able to reconstitute *de novo* PE synthesis (Figure 23B). Hence, the *in vivo* results found for the hCEPT1 mutants were consistent with the *in vitro* assessment of their CDP-aminoalcohol specificities, further supporting the hypothesis of a role for glycine^{156} in CDP-ethanolamine binding.

4. Identification of putative DAG binding residues

Chimeric enzyme studies of the *S. cerevisiae CPT1* and *EPT1* gene products have identified a linear region of 218 amino acids sufficient to confer DAG acyl-chain specificity (Hjelmstad et al., 1994). As DAG is found in the membrane, in the predicted model this region would encompass the first two membrane-spanning helices of the protein. Recently, an analysis of the invariant amino acids from various acyltransferases identified a region that is important in binding the glycerol-3-phosphate substrate in the *sn*-glycerol-3-phosphate acyltransferase (GPAT) reaction (Lewin et al., 1999). This region of GPAT (Block III) shares some identity with the second predicted membrane-spanning helix of hCEPT1p. Because GPAT adds an acyl group and this product would be similar in structure to diacylglycerol, this region was chosen to attempt to identify residues involved in the DAG binding of hCEPT1p (Figure 24A). A number of residues spanning the entire predicted helix were targeted for site-directed mutagenesis, including glutamate^{215}, which aligns with a glutamate that was demonstrated to be important for binding glycerol-3-phosphate by GPAT. All mutations were constructed using the plasmid pAH9 as a template, which is a yeast expression vector directing the high-copy

Figure 23. Metabolic labelling of hCEPT1 CDP-alcohol specificity mutants expressed in *S. cerevisiae* HJ091. Radiolabelled choline (A) or ethanolamine (B) was added to exponential-phase HJ091 cells expressing the hCEPT1 CDP-alcohol specificity mutants from pAH9 for 1 hour at 30°C. Total uptake and the incorporation of radiolabel into phospholipids was monitored and quantified as described in Materials and Methods. Results are shown as the mean for at least four separate experiments.

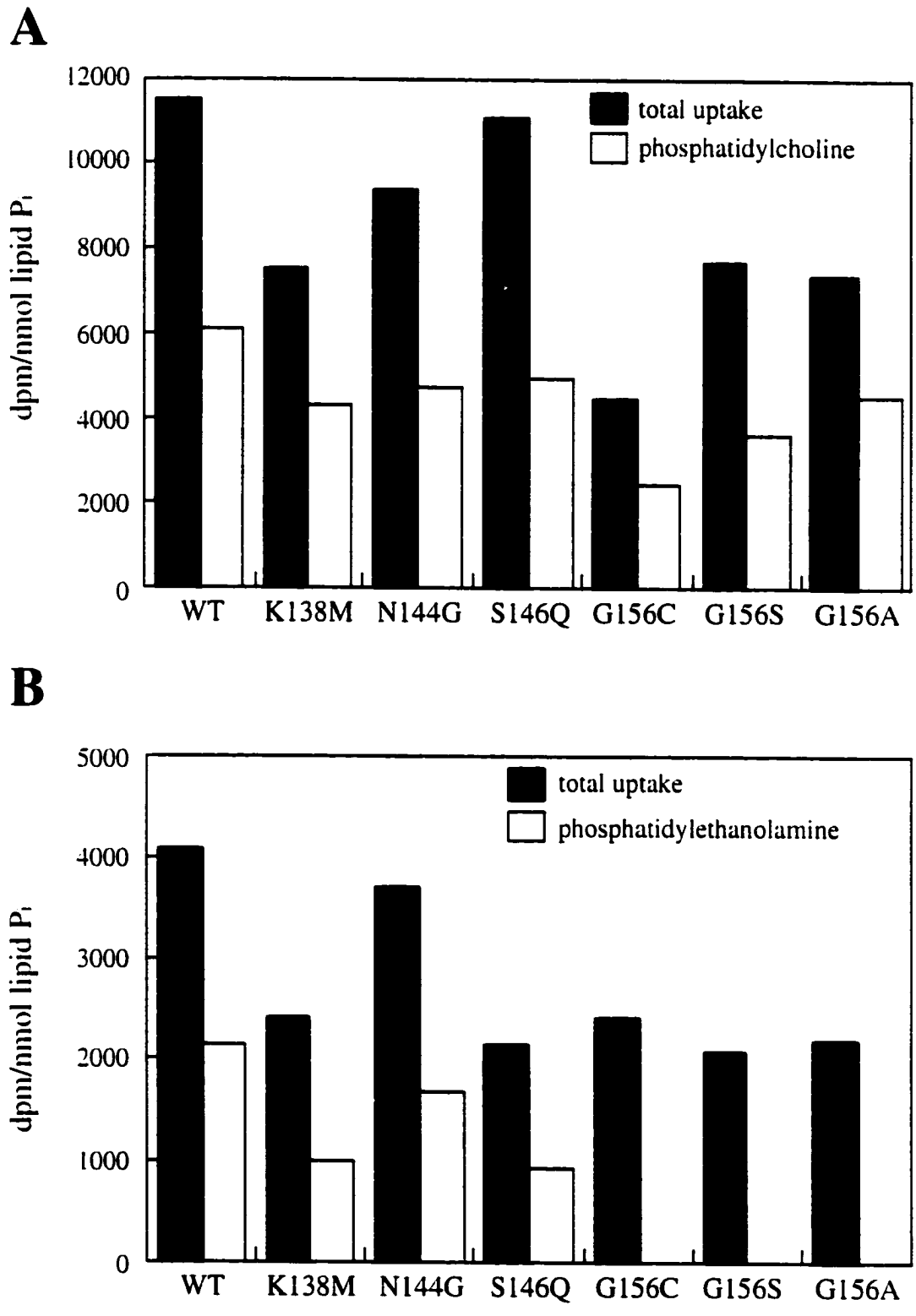

A
12000
10000
8000
6000
4000
2000
0
dpm/nmol lipid Pi
total uptake
phosphatidylcholine
WT
K138M
N144G
S146Q
G156C
G156S
G156A
B
5000
4000
3000
2000
1000
0
dpm/nmol lipid Pi
total uptake
phosphatidylethanolamine
WT
K138M
N144G
S146Q
G156C
G156S
G156A

Figure 23

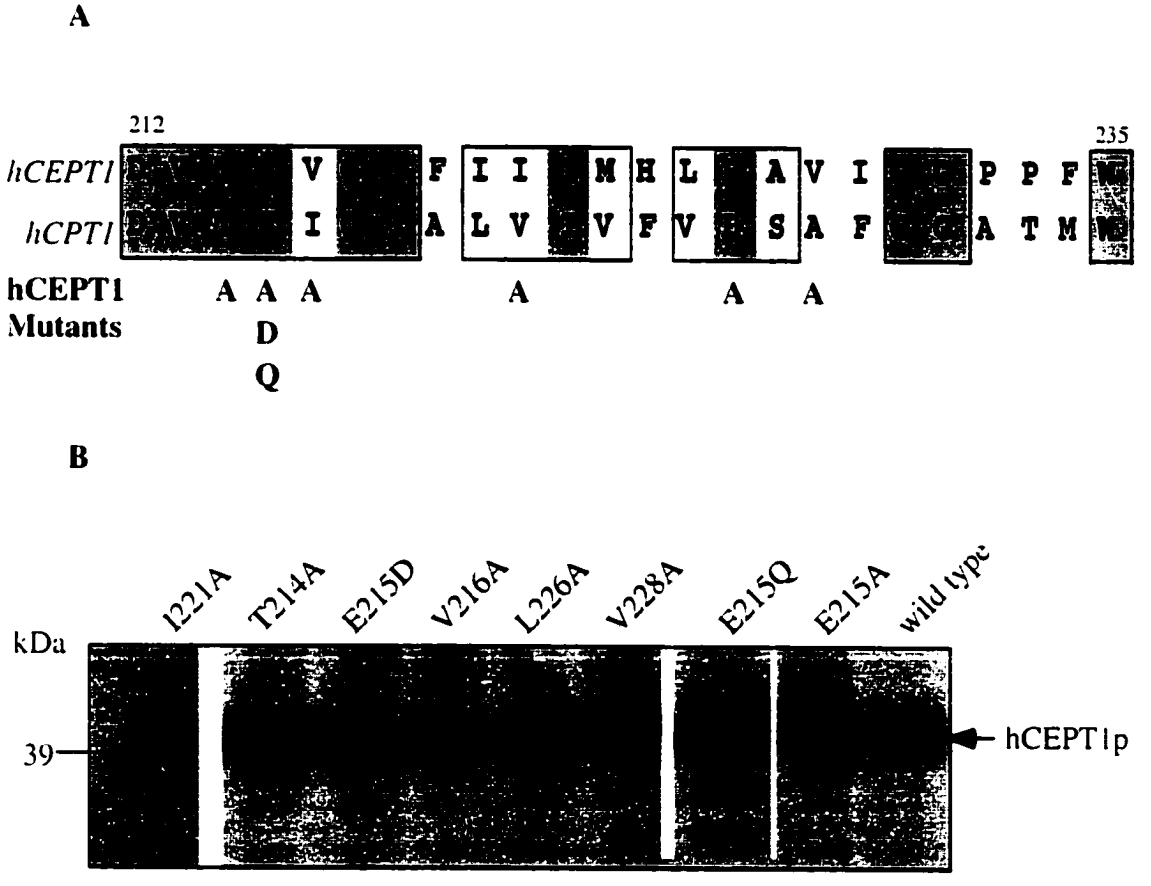


Figure 24. Identification of residues involved in diacylglycerol substrate specificity. (A) Alignment of the second predicted membrane-spanning helix from human CPTs. Site-directed mutants of hCEPT1 used in this work are indicated in bold below. Dark shading indicates identical residues and light shading indicates conserved residues. (B) Western blot of T7-tagged hCEPT1 site-directed mutants expressed in *S. cerevisiae* strain HJ091 (*cpt1::LEU2 ept1*) using the T7-HRP antibody (1:5000).

expression of a T7-tagged version of hCEPT1p.

5. Expression and *in vitro* activity of site-directed mutants

pAH9 plasmids carrying the various mutations were transformed into *S. cerevisiae* strain HJ091 (*cpt1::LEU2 ept1*), which ensured that any CPT or EPT activity detected would be plasmid-encoded. Western blot analysis of HJ091 membrane fractions using the T7-HRP antibody established that similar levels of full-length hCEPT1 protein were being expressed in each case (Figure 24B).

The cholinephosphotransferase activities of hCEPT1 mutants were determined *in vitro* using the sodium cholate mixed micelle assay, which ensured uniform delivery of each DAG substrate. Each hCEPT1 mutant was analyzed for CPT activity using a wide variety of DAGs and the results are displayed in Table 5. A number of the mutations, namely T214A, V216A, and I221A, altered the profile of DAG utilization when compared to that of wild-type hCEPT1p, without completely abolishing CPT activity. This suggests that these residues may be important in determining DAG specificity, although their exact role cannot be elucidated simply from these *in vitro* results. However, the E215A, E215D, and E215Q mutations dramatically reduced the CPT activity of hCEPT1p regardless of the DAG used as a substrate. This suggests that glutamate[215] may have a role in general DAG binding, and not in determining the DAG specificity of hCEPT1p.

In contrast, the mutations L226A and V226A did not significantly alter the CPT activity of hCEPT1p when compared to wild-type. Hence, these residues do not seem to play a role in DAG binding or specificity. However, these results do help to position one of the predicted membrane-spanning helices of hCEPT1p. Depending on the algorithm

Table 5. *In vitro* cholinephosphotransferase activity of DAG mutants

DAG Species	CPT Activity (nmol min^{-1} mg^{-1})								
	Mutant								
	WT	T214A	E215A	E215D	E215Q	V216A	I221A	L226A	V228A
di18:1	10.46	6.74	0.38	0.44	0.14	6.20	1.95	11.03	9.08
di10:0	7.07	0.19	0.16	0.16	0.13	3.92	0.15	5.57	6.64
di12:0	1.26	0.17	0.11	0.15	0.11	2.45	0.15	2.57	2.00
di14:0	1.26	0.17	0.10	0.11	0.10	2.12	0.14	3.03	1.33
di16:0	0.63	0.16	0.15	0.15	0.13	0.24	0.14	0.38	0.25
di16:1	16.76	5.51	0.12	0.17	0.10	3.17	1.32	13.14	12.37
16:0/18:1	11.74	6.20	0.18	0.25	0.14	4.31	1.33	12.52	10.21
16:0/22:6	13.30	5.51	0.23	0.43	ND[1]	4.71	1.74	13.22	10.45
PAF Precursor	5.24	0.26	0.13	0.13	0.13	2.14	0.41	5.12	4.22

[1]ND: not determined

Choline- and ethanolaminephosphotransferase activities were determined in microsomal preparations from *S. cerevisiae* HJ091 (*cpt1::LEU2 ept1*) expressing hCEPT1 DAG specificity mutants from pAH9 using the sodium cholate mixed micelle assay as described in Materials and Methods. Results are shown as the average of at least four experiments.

used, two different models for the structure of hCEPT1p are predicted (Figure 25). In one model (A), the third predicted membrane-spanning helix contains the residues that were mutated in the present study, while in the second model (B), the residues are contained within the second predicted membrane-spanning helix. The arrow in each model identifies the helix in question. The catalytic mechanism for CPT does not appear to go through an enzyme-bound intermediate; therefore, the two substrates (DAG and CDP-choline) would need to be in close proximity to each other to undergo the reaction. Therefore, it could be predicted that residues mutated near the cytoplasmic side of the membrane-spanning helix would have an effect on enzymatic activity, while residues mutated on the opposite side of the helix, away from the catalytic site, would have little effect. The *in vitro* results (Table 5) suggest that residues 226 and 228 are located on the opposite side of the helix, away from the catalytic site, because mutation of either to alanine had no significant effects on CPT activity, while mutations in residues 214, 215, 216, and 221 reduced activity or altered DAG specificity. Based on these results, it is predicted that the model proposed in Figure 25B is correct with regards to the second membrane-spanning helix.

6. *In vivo* activity of site-directed mutants

We also tested the ability of the pAH9 plasmids carrying the DAG specificity mutations *in vivo*, through expression in *S. cerevisiae* HJ091 (*cpt1::LEU2 ept1*) and the ability of each mutant to reconstitute the CDP-choline pathway for the synthesis of PC and the CDP-ethanolamine pathway for the synthesis of PE *via* the addition of radiolabelled choline or ethanolamine, respectively. All yeast expressing hCEPT1 mutant proteins demonstrated wild-type levels of choline uptake, and all but the E215A mutation

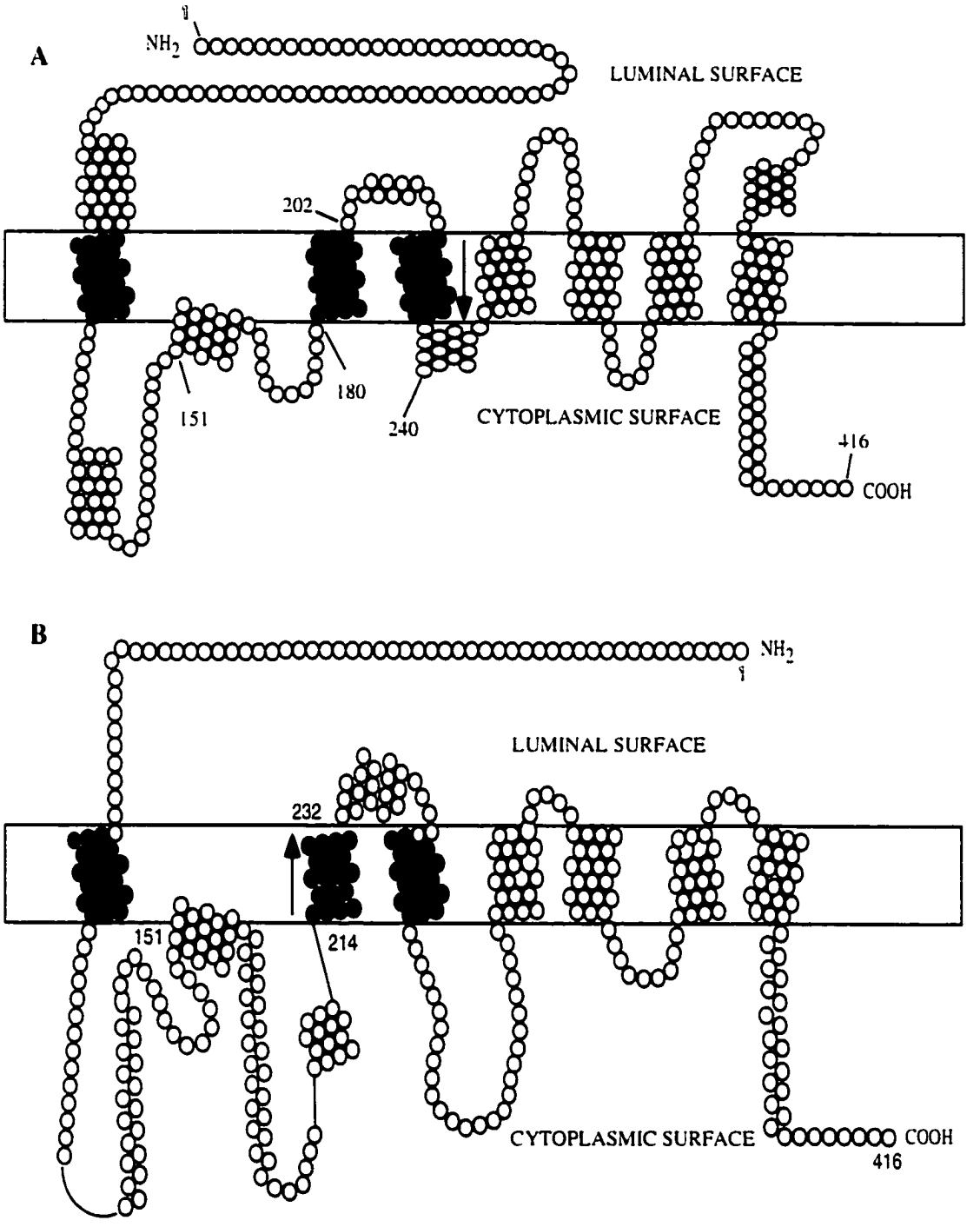


Figure 25. Two predicted models of hCEPT1p membrane topography. The direction of the predicted DAG binding helix is indicated by the arrow

synthesized PC at wild-type levels (Figure 26A). Significantly, hCEPT1 proteins containing either the E215Q or E215D mutations, which both had extremely low *in vitro* CPT activity, were able to reconstitute PC synthesis to wild-type levels *in vivo*. These results are consistent with earlier studies that showed that CPT is not the rate-limiting step in the *de novo* biosynthesis of PC. The E215A mutation was unable to reconstitute PC biosynthesis *in vivo*, thereby correlating with the *in vitro* results, which demonstrated that very low CPT activity was associated with the E215A mutant. Mutation to alanine is a dramatic change and these results indicate that either the size or charge (or both) at residue 215 is required for the CPT activity of hCEPT1p. Metabolic labelling with ethanolamine demonstrated that all of the yeast expressing hCEPT1 mutant proteins were capable of taking up ethanolamine to wild-type levels, and all but the E215 mutations were able to reconstitute *de novo* PE synthesis (Figure 26B). This is interesting in light of the fact that both E215D and E215Q are capable of reconstituting PC biosynthesis to wild-type levels (Figure 26A). This suggests that glutamate^{215} may also play a role in CDP-aminoalcohol specificity, although the exact role remains to be elucidated. The *in vivo* results also confirm the *in vitro* observation that mutations in residues 226 and 228 have no effect on the ability of hCEPT1p to synthesize PC.

C. Human cholinephosphotransferase 1 (hCPT1)

1. Cloning of hCPT1 and analysis of hCPT1p

Previous structure/function analysis of the yeast cholinephosphotransferase (*CPT1*) and choline/ethanolaminephosphotransferase (*EPT1*) enzymes resulted in several predictions. The first three membrane-spanning helices were the maximum region required for diacylglycerol (DAG) binding, and the first cytoplasmic loop was required

Figure 26. Metabolic labelling of hCEPT1 diacylglycerol specificity mutants expressed in *S. cerevisiae* HJ091. Radiolabelled choline (A) or ethanolamine (B) was added to exponential-phase HJ091 cells expressing the hCEPT1 DAG specificity mutants from pAH9 for 1 hour at 30°C. The incorporation of radiolabel into phospholipids, together with the metabolites of the CDP-choline and CDP-ethanolamine pathways, was monitored and quantified as described in Materials and Methods. Results are shown as the mean for at least four separate experiments.

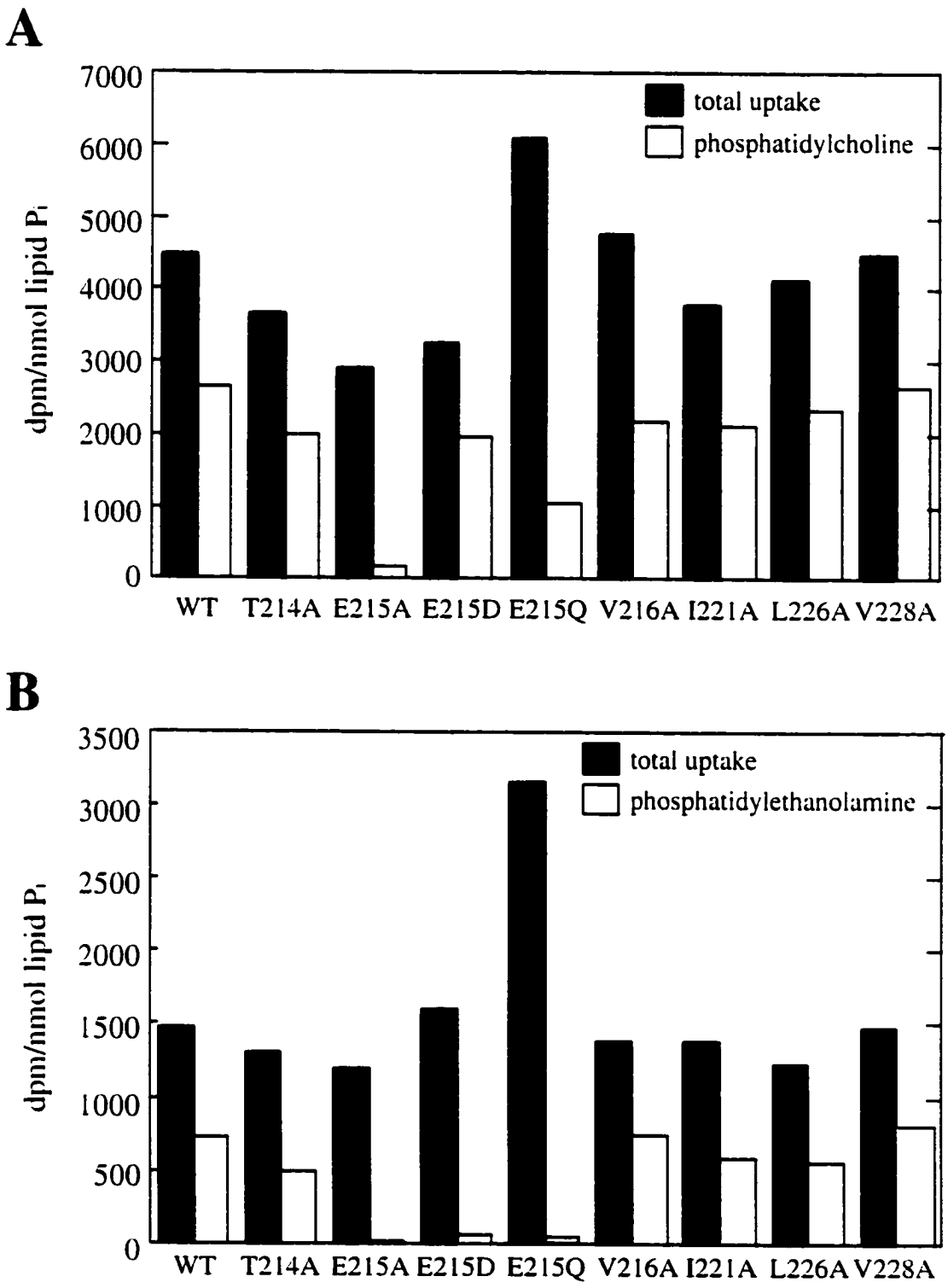

A
7000
6000
5000
4000
3000
2000
1000
0
dpm/nmol lipid Pi
total uptake
phosphatidylcholine
WT T214A E215A E215D E215Q V216A I221A L226A V228A
B
3500
3000
2500
2000
1500
1000
500
0
dpm/nmol lipid Pi
total uptake
phosphatidylethanolamine
WT T214A E215A E215D E215Q V216A I221A L226A V228A

Figure 26

for CDP-alcohol binding (Hjelmstad et al., 1994). Within the CDP-alcohol binding region of *CPT1* and *EPT1*, and also present in several other phospholipid synthesizing enzymes that catalyze the displacement of a CDP-alcohol by a second alcohol for the formation of a phosphoester bond, is a CDP-alcohol phosphotransferase motif, $DG(x)_2AR(x)_8G(x)_3D(x)_3D$. Site-directed mutagenesis studies predict this motif performs catalysis by employing the final two aspartate residues in a general base reaction that does not proceed through an enzyme-bound intermediate (Williams and McMaster, 1998). A search of the expressed sequence tag (EST) data base for cDNAs with similarity to the yeast Cpt1p CDP-alcohol phosphotransferase motif revealed two classes of mammalian cDNAs. An alignment of all of the members of one class revealed that one of the human cDNAs was full length (hCEPT1) and this cDNA was described earlier. Alignment of the members of the second class of mammalian cDNAs with similarity to the yeast Cpt1p CDP-alcohol phosphotransferase motif revealed that none of the cDNAs were full length. Previous work in this laboratory extended the longest of these partial cDNAs by an additional 322 base pairs by two successive 5' rapid amplification of cDNA ends (RACE) protocols. As part of this study, a human brain cDNA library was screened using an oligonucleotide derived from the most 5' end of the farthest RACE extension, resulting in the isolation of five cDNAs of sufficient length to code for a full-length cholinephosphotransferase protein. Each of these cDNAs was sequenced in its entirety and found to contain an insert of 1505-1532 base pairs (including poly(A) tails), indicating the cDNAs extend through to the 3' end of the mRNA transcript (Figure 27). Three of the cDNAs code for a protein of 407 amino acids (Figure 28A: hCPT1, Genbank™ accession number AF195623). The full-length hCPT1 amino acid sequence is

ATGGCGGCAG GCGCCGGGGC CGGGTCCGCG CCGCGCTGGC TGAGGGCGCT GAGCGAGCCG CTGAGCGCGG

CGCAGCTGCG GCGACTGGAG GAGCACCGCT ACAGCGCGGC GGGCGTCTCG CTGCTCGAGC CGCCGCTGCA

GCTCTACTGG ACCTGGCTGC TCCAGTGGAT CCCGCTCTGG ATGGCCCCCA ACTCCATCAC CCTGTTGGGG

CTCGCCGTCA ACGTGGTCAC CACGCTCGTG CTCATCTCCT ACTGTCCCAC GGCCACCGAA GAGGCACCAT

ACTGGACATA CCTTTTATGT GCACTGGGAC TTTTTATTTA CCAGTCACTG GATGCTATTG ATGGGAAACA

AGCCAGAAGA ACAAACTCTT GTTCCCCTTT AGGGGAGCTC TTTGACCATG GCTGTGACTC TCTTTCCACA

GTATTTATGG CAGTGGGAGC TTCAATTGCC GCTCGCTTAG GAACTTATCC TGACTGGTTT TTTTCCTGCT

CTTTTATTGG GATGTTTGTG TTTTATTGCG CTCATTGGCA GACTTATGTT TCAGGCATGT TGAGATTTGG

AAAAGTGGAT GTAACTGAAA TTCAGATAGC TTTAGTGATT GTCTTTGTGT TGTCTGCATT TGGAGGAGCA

ACAATGTGGG ACTATACGAT TCCTATTCTA GAAATAAAAT TGAAGATCCT TCCAGTTCTT GGATTTCTAG

GTGGAGTAAT ATTTTCCTGT TCAAATTATT TCCATGTTAT CCTCCATGGT GGTGTTGGCA AGAATGGATC

CACTATAGCA GGCACCAGTG TCTTGTCACC TGGACTCCAC ATAGGACTAA TTATTATACT GGCAATAATG

ATCTATAAAA AGTCAGCAAC TGATGTGTTT GAAAAGCATC CTTGTCTTTA TATCCTAATG TTTGGATGTG

TCTTTGCTAA AGTCTCACAA AAATTAGTGG TAGCTCACAT GACCAAAAGT GAACTATATC TTCAAGACAC

TGTCTTTTTG GGGCCAGGTC TTTTGTTTTT AGACCAGTAC TTTAATAATT TTATAGACGA ATATGTTGTT

CTATGGATGG CAATGGTGAT TTCTTCATTT GATATGGTGA TATACTTTAG TGCTTTGTGC CTGCAAATTT

CAAGACACCT TCATCTAAAT ATATTCAAGA CTGCATGTCA TCAAGCACCT GAACAGGTTC AAGTTCTTTC

TTCAAAGAGT CATCAGAATA ACATGGATTG AAGAGACTTC CGAACACTTG CTATCTCTTG CTGCTGCTGT

TTCATGGAAG GAGATATTAA ACATTTGTTT AATTTTTATT TAAGTGTTAT ACCTATTTCA GCAAATAAAA

TATTTCATTG CTTAAAAAAA AAAAAAAAAA AAAAAA

Figure 27. Nucleotide sequence of Gene Trapper cDNAs (hCPT1). Five clones isolated from a human brain cDNA library were sequenced in their entirety on both strands. The open reading frame corresponding to hCPT1 is underlined. Two of the five clones contained a 27-base pair insert at nucleotide 631.

A

M A A G A G A G S A P R W L R A L S E P L S A A Q L R R L E E H R Y S
A A G V S L L E P P L Q L Y W T W L L Q W I P L W M A P N S I T L L G
L A V N V V T T L V L I S Y C P T A T E E A P Y W T Y L L C A L G L F
I Y Q S L D A I D G K Q A R R T N S C S P L G E L F D H G C D S L S T
V F M A V G A S I A A R L G T Y P D W F F S C S F I G M F V F Y C A H
W Q T Y V S G M L R F G K V D V T E I Q I A L V I V F V L S A F G G A
T M W D Y T I P I L E I K L K I L P V L G F L G G V I F S C S N Y F H
V I L H G G V G K N G S T I A G T S V L S P G L H I G L I I I L A I M
I Y K K S A T D V F E K H P C L Y I L M F G C V F A K V S Q K L V V A
H M T K S E L Y L Q D T V F L G P G L L F L D Q Y F N N F I D E Y V V
L W M A M V I S S F D M V I Y F S A L C L Q I S R H L H L N I F K T A
C H Q A P E Q V Q V L S S K S H Q N N M D - **406**

B

M A A G A G A G S A P R W L R A L S E P L S A A Q L R R L E E H R Y S
A A G V S L L E P P L Q L Y W T W L L Q W I P L W M A P N S I T L L G
L A V N V V T T L V L I S Y C P T A T E E A P Y W T Y L L C A L G L F
I Y Q S L D A I D G K Q A R R T N S C S P L G E L F D H G C D S L S T
V F M A V G A S I A A R L G T Y P D W F F S C S F I G M F V F Y C A H
W Q T Y V S G M L R F G K V D V T E I Q I A L V I V F V L S A F G G A
T M W D Y T F S - **218**

Figure 28. Predicted amino acid sequence of hCPT1p and hCPT1βp. (A) Amino acid sequence of hCPT1 (Genbank™ accession number AF195623) and (B) Amino acid sequence of hCPT1β (Genbank™ accession number AF195624). The CDP-aminoalcohol phosphotransferase motif is underlined.

60% identical to hCEPT1 (Figure 29) and Kyte-Doolittle hydropathy plots predict proteins with very similar secondary structures (Figure 30). An inspection of the hCPT1 and hCPT1β amino acid sequences revealed the presence of the CDP-alcohol phosphotransferase motif at amino acids 114-136. As with hCEPT1p, there are an estimated seven membrane-spanning helices in hCPT1p.

Two of the cDNAs isolated from the human brain library colony screen contained an identical cDNA to hCPT1 except for an insert of 27 base pairs (corresponding to predicted amino acid 217) that result in a termination at position 219 (Figure 28B: hCPT1β, Genbank™ accession number AF195624). The hCPT1β protein consists of the CDP-alcohol binding motif and the first two predicted membrane-spanning helices, and thus possesses only the catalytic portion of the enzyme.

Similarity searches of public databases were used to determine the genomic location of hCPT1. The hCPT1 gene was positioned to chromosome 12q between microsatellite markers D12S346 and D12S78 and a BAC clone from the human genome sequencing project contains the entire hCPT1 gene sequence and flanking DNA (Genbank accession number AC010205). The hCPT1 gene is 32 kb in length and is comprised of 9 exons (Figure 31A). An analysis of the hCPT1 gene indicated that the extra DNA sequence found in the hCPT1β cDNAs is contained within the hCPT1 gene (exon 4a), implying the hCPT1β cDNA isolated is a true splice variant. To further validate if hCPT1β represents a hCPT1 splice variant, several different human cDNA libraries were subjected to PCR analysis using primers that span the predicted exon insertion site of hCPT1β (Figure 31B). Each of the libraries possessed cDNAs coding for hCPT1 and the hCPT1β splice variant. To determine if the hCPT1β cDNA was capable of producing a protein, a T7-

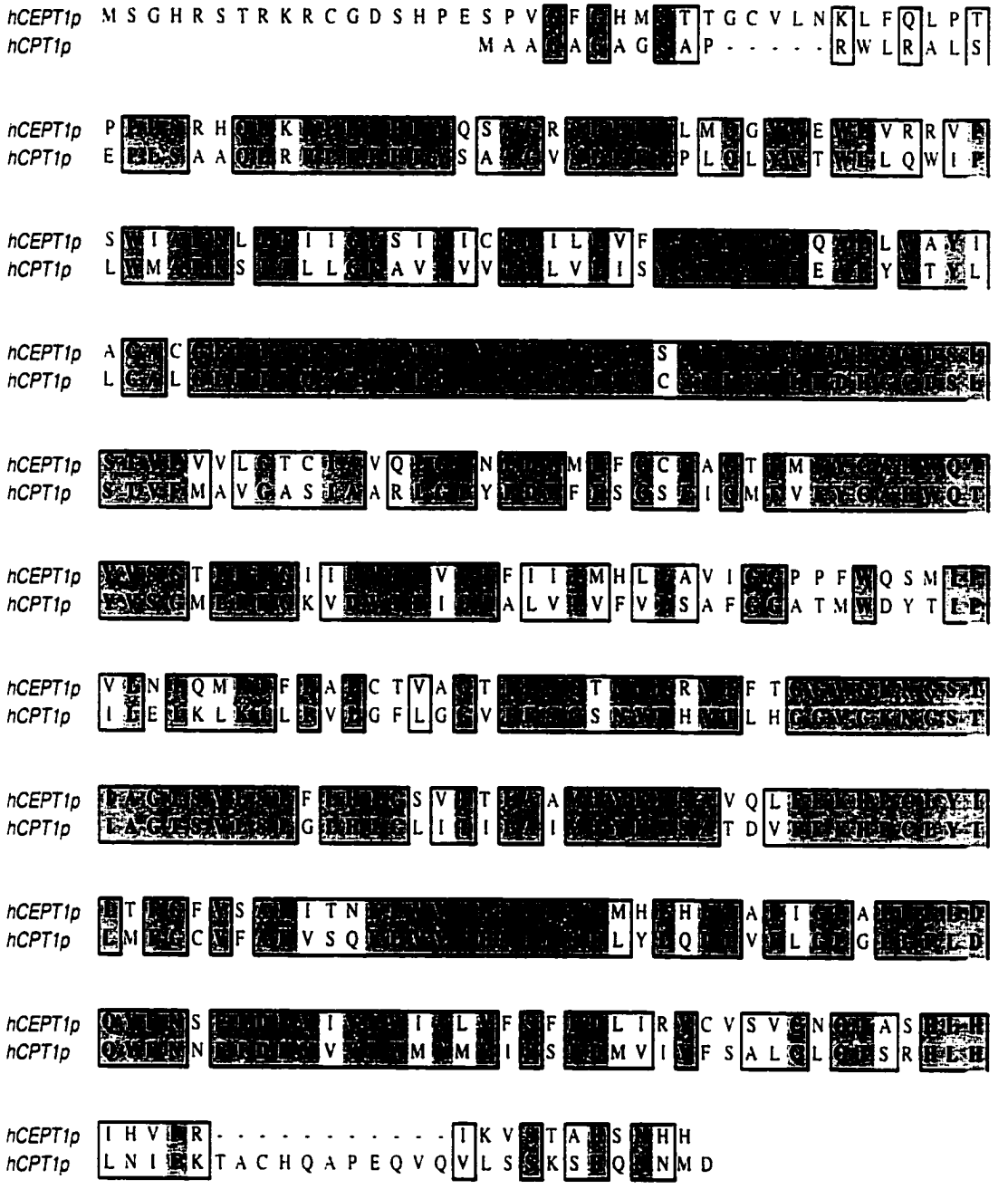


Figure 29. Alignment of hCPT1p with hCEPT1p. The amino acid sequences of hCPT1p and hCEPT1p were aligned using CLUSTALW. Dark shading indicates identical residues and light shading indicates conserved residues.

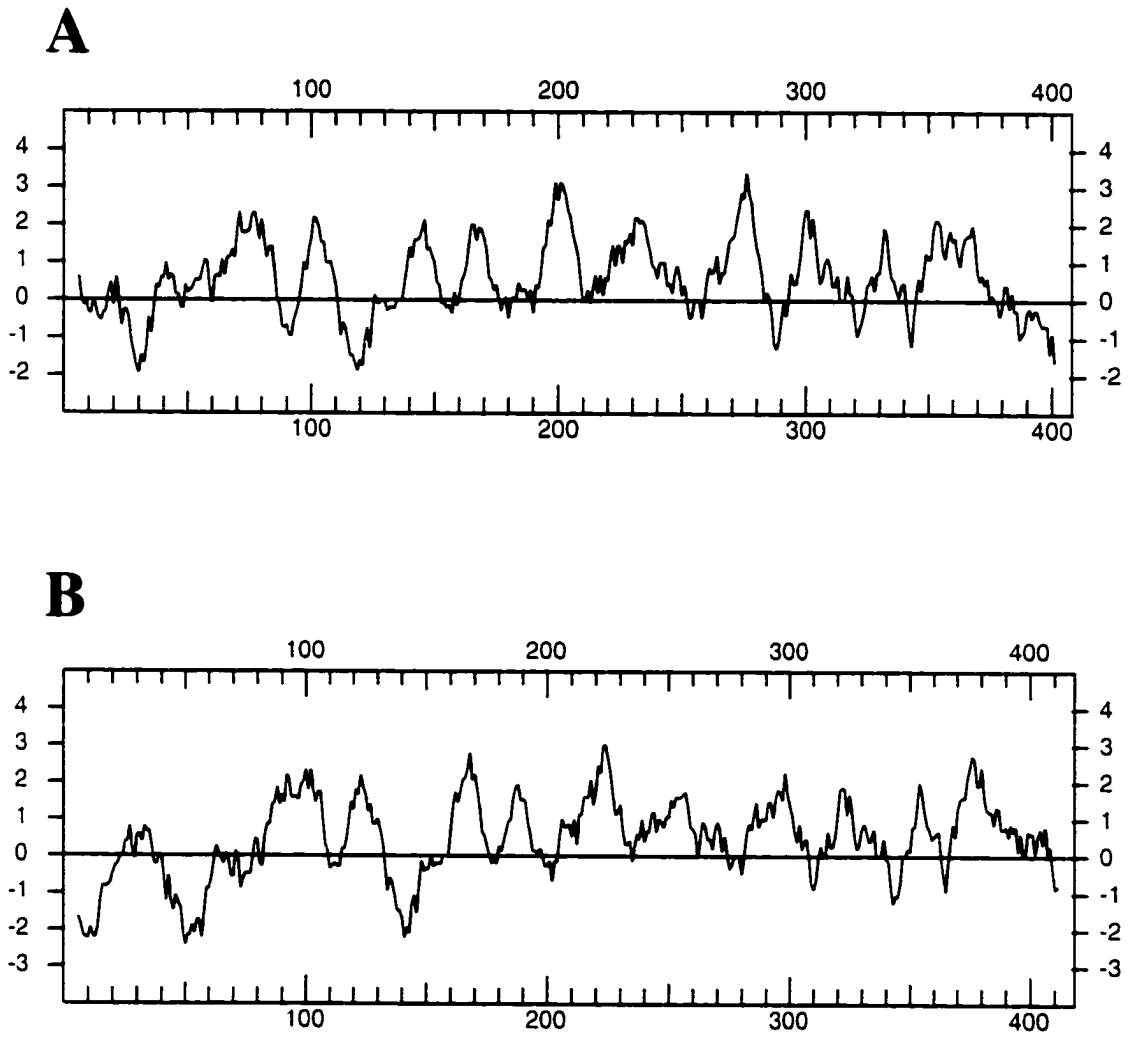
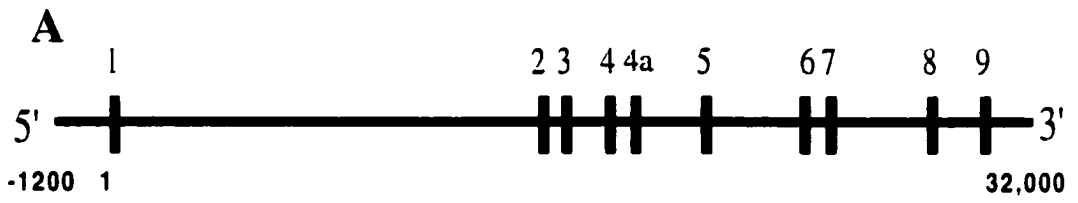
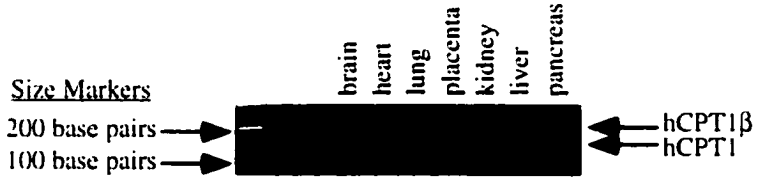
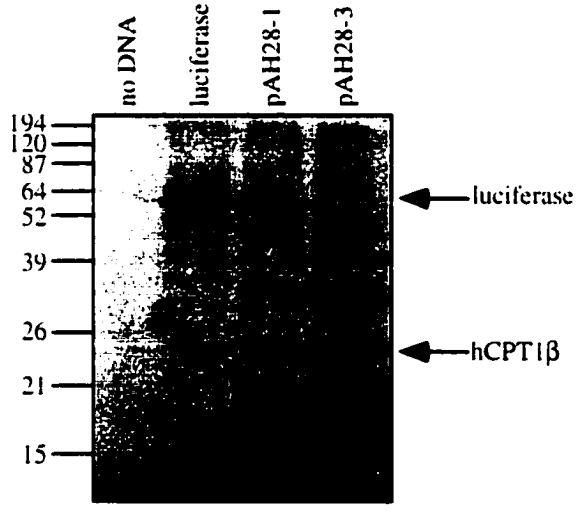


Figure 30. Predicted secondary structure of hCPT1p. Kyte-Doolittle hydropathy plots for (A) hCPT1p and (B) hCEPT1p.

Figure 31. Structure of the hCPT1 gene and analysis of hCPT1 splice variants. (A) The predicted intron/exon splice junctions of the hCPT1 gene (exon width not to scale). (B) PCR analysis of cDNA libraries generated from indicated human tissues using primers that span the predicted hCPT1β exon. The hCPT1-specific primers used were 5'-GAACAGGAAAATATTACTCCACC and 5'-GCTTTAGTGATTGTCTTTGTGTTG. Amplification of the hCPT1 cDNA would result in a DNA fragment of 134 base pairs whereas the hCPT1β cDNA would produce a 161-base pair fragment. (C) *In vitro* transcription/translation of hCPT1β from the T7 promoter (pAH28) using the Promega kit as described in Materials and Methods. (D) Western blot of T7-tagged hCPT1β (pAH28) transiently transfected into CHO-K1 cells. Subcellular fractions were prepared and 20 μg of either cytosol (C) or membrane (M) fractions were subjected to 10% SDS-PAGE and immunoblotting with T7-HRP (1:5000).


A
1
2 3
4 4a
5
6 7
8
9
5'
3'
-1200
1
32,000


B
brain
heart
lung
placenta
kidney
liver
pancreas
Size Markers
200 base pairs
100 base pairs
hCPT1β
hCPT1


C
no DNA
luciferase
pAH28-1
pAH28-3
194
120
87
64
52
39
26
21
15
luciferase
hCPT1β

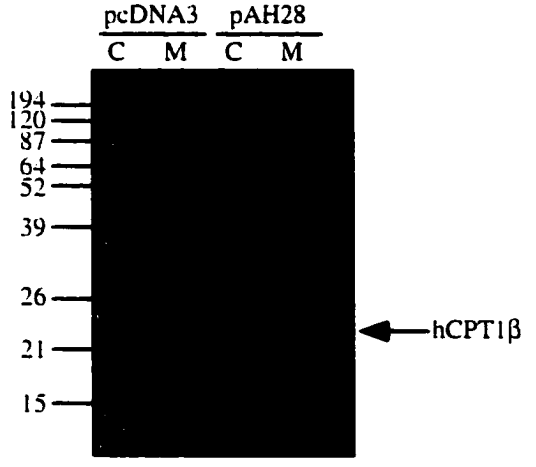

D
pcDNA3
pAH28
C
M
C
M
194
120
87
64
52
39
26
21
15
hCPT1β

Figure 31

tagged version of hCPT1β was cloned into the mammalian expression vector pcDNA3 (pAH28). We used *in vitro* transcription/translation to direct expression of hCPT1β from the T7 promoter of pcDNA3 and demonstrated that pAH28 was able to synthesize a protein of approximately 24 kDa, which is the predicted molecular mass (Figure 31C). Further, we transiently transfected pAH28 into CHO-K1 cells and detected a protein of approximately 24 kDa in the membrane fraction (Figure 31D). Unfortunately, no enzyme activity could be detected above background CPT levels when di18:1 DAG was used as a substrate. It remains to be seen whether hCPT1β codes for an active enzyme.

2. Expression and enzymatic characterization of hCPT1p

To enzymatically assess hCPT1 substrate specificities, the hCPT1 coding region was inserted in frame with a T7-epitope tag (pAH12) for expression in the *S. cerevisiae* strain HJ091 (*cpt1::LEU2 ept1*). This yeast is devoid of its endogenous CPT and EPT activities due to inactivation of the genomic loci coding for these activities. This ensured any enzyme activity observed was derived from the transformed hCPT1 coding sequence. The hCPT1 protein was successfully expressed in *S. cerevisiae* as a full-length protein of approximately 39 kDa as determined by Western blot analysis, albeit at levels significantly lower than that for hCEPT1 expressed from the identical vector (Figure 32).

There are several commonly used assays for measurement of mammalian CPT and EPT activities. The assays are very similar except for (i) the nature in which the DAG substrate is solubilized for subsequent delivery in the assay mix and (ii) the use of Mg^{2+} or Mn^{2+} as the essential cation. We tested the delivery of DAG in Tween 20 emulsions (O et al., 1989), a deoxycholate emulsion (Sleight and Kent, 1980), complexed with PC in liposomes (Cornell, 1992), in Triton X-100 mixed micelles (Hjelmstad and Bell, 1992),

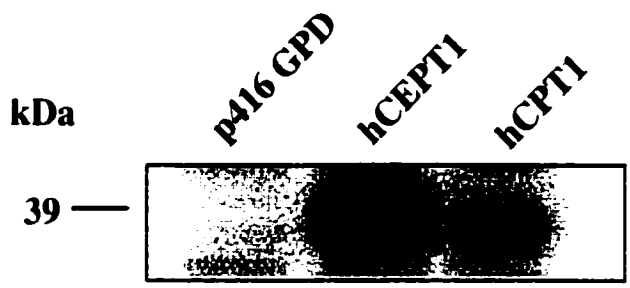


Figure 32. Western blot of hCPT1 expressed in *S. cerevisiae* HJ091. The hCPT1 and hCEPT1 coding sequences were spliced in frame with a T7-epitope tag at their N-termini (pAH12 and pAH9, respectively) and expressed from the constitutive GPD promoter in HJ091 (*cpt1::LEU2 ept1*) yeast. Membrane preparations (20 µg of protein) were separated by 12% SDS-PAGE and blotted with T7-HRP (1:5000) as described in Materials and Methods.

or in sodium cholate mixed micelles (Bru et al., 1993), using either Mg^{2+} or Mn^{2+} as essential cation donor. We first determined the best method for delivery of DAG to hCPT1 (Table 6). The highest enzyme activity was observed using DAG delivery in either a very small amount of Tween 20 (0.004%) or in PC liposomes. We were unable to detect EPT activity with any of the diradylglycerol species tested, under each of the assay conditions employed. Interestingly, there was no enzymatic activity for hCPT1p using the sodium cholate mixed micelle assay, which was an assay used to characterize the hCEPT1 protein and site-directed mutants (Tables 4 and 5). We chose to characterize hCPT1 further using the Tween 20 (0.004%) assay conditions as (i) this was the assay originally used in the characterization of hCEPT1 activity, and (ii) this assay was also used to demonstrate the existence of a putative microsomal PAF-specific CPT activity.

We also determined the Mg^{2+} and Mn^{2+} requirement for hCPT1-derived CPT activity using the Tween 20 assay and an essential requirement for either cation was observed (Figure 33). However, Mg^{2+} activated CPT activity to levels 10-fold higher than did Mn^{2+}. The specificity of the DAG donor was also determined in the presence of either Mg^{2+} or Mn^{2+} (Table 7). In general, Mg^{2+}-derived activities were higher for most substrates than those obtained using Mn^{2+} as the essential cation. The DAG specificity of the Mg^{2+}-derived activities was di18:1 > 16:0/22:6 = 16:0/18:1 > 18:0/20:4 > di16:0. None of the other DAGs tested were capable of supporting hCPT1-derived CPT activity in the presence of Mg^{2+}. When Mn^{2+} was the cation included in the assay the DAG specificity was substantially altered with di10:0 = 18:1/2:0 > 16:0/18:1 > 16:0/22:6 = di18:1 > di16:0 > di14:1 = di16:1. Notably, there was no activity when diradylglycerols

Table 6. *In vitro* cholinephosphotransferase activity of hCPT1p using various assays

Assay	CPT Activity (nmol min^{-1} mg^{-1})
Tween 20 (0.004%)	0.419
Tween 20 (0.1%)	0.165
Deoxycholate (0.1%)	ND[1]
PC liposome	0.588
Triton X-100 (mixed micelle)	ND
Sodium cholate (mixed micelle)	ND

[1]ND: not detectable

Cholinephosphotransferase activity was determined in microsomal preparations from *S. cerevisiae* HJ091 (*cpt1::LEU2 ept1*) expressing hCPT1 from pAH12. The assays used are described in detail in Materials and Methods. Results are shown as the average from at least four experiments.

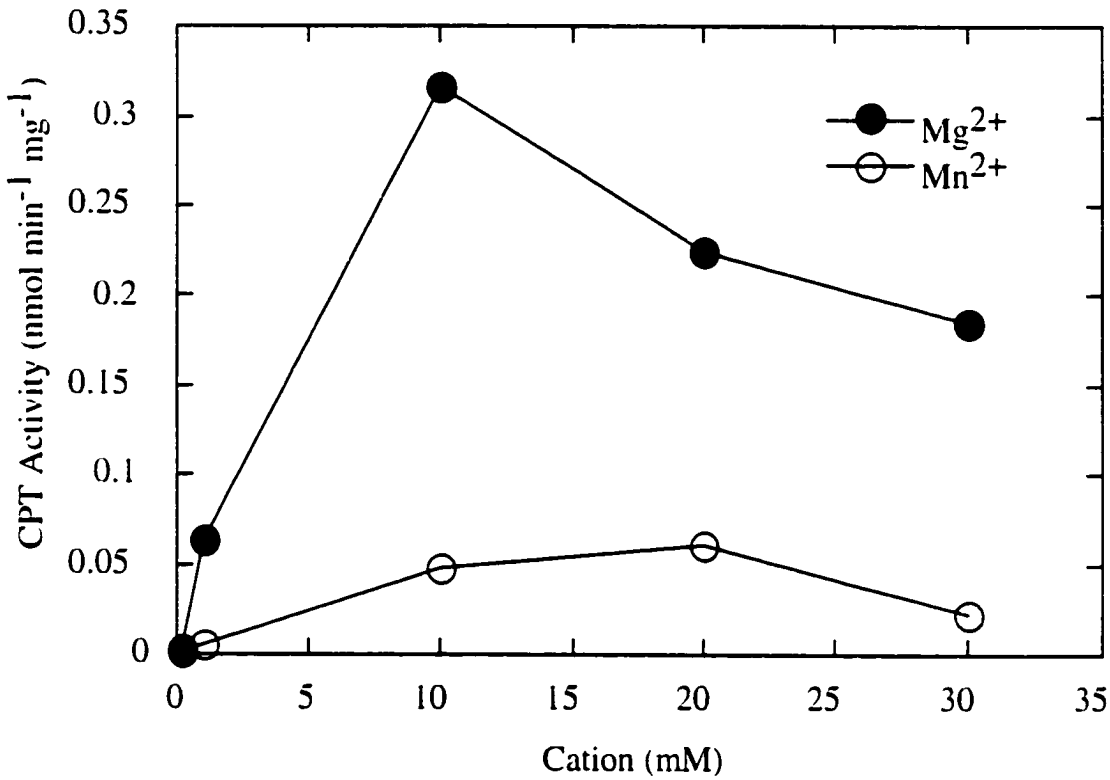


Figure 33. Cation requirements of hCPT1p. Enzyme activities were determined using the Tween 20 (0.004%) assay with di18:1 diacylglycerol as described in Materials and Methods from microsomal membranes of HJ091 yeast expressing pAH12 (hCPT1).

Table 7. *In vitro* cholinephosphotransferase activity of hCPT1p

Diradylglycerol substrate	CPT activity (nmol min^{-1} mg^{-1})	
	Mg^{2+}	Mn^{2+}
di8:0	ND[1]	ND
di10:0	ND	0.282
di12:0	ND	ND
di14:0	ND	0.005
di16:0	0.040	0.022
di16:1	ND	0.003
di18:1	0.419	0.042
16:0/18:1	0.248	0.102
16:0/22:6	0.251	0.049
18:0/20:4	0.168	ND
ceramides	ND	ND
16:0(O)/2:0	ND	ND
16:0(O)/20:4	ND	ND
18:1/2:0	ND	0.243

[1]ND: not detectable

Cholinephosphotransferase activity was determined in microsomal preparations of *S. cerevisiae* HJ091 (*cpt1::LEU2 ept1*) expressing hCPT1 from pAH12 using the Tween 20 (0.004%) assay as described in Materials and Methods. Results are shown as the average of at least four experiments.

capable of synthesizing PAF (16:0(*O*)/2:0) or PAF precursor (16:0(*O*)/20:4) were used as substrates for hCPT1.

A PAF-specific cholinephosphotransferase activity detected in crude subcellular extracts was previously differentiated from PC-specific activity based on DTT sensitivity. The PAF-specific activity was slightly stimulated by DTT while the PC-specific activity was greatly inhibited by DTT. We repeated these experiments using the cloned hCPT1 as specific enzyme sources. Low concentrations of DTT moderately increased the activity of hCPT1 for the synthesis of PC and surprisingly allowed hCPT1 to synthesize PAF (Figure 34). Hence, hCPT1 does have the capacity to synthesize PAF *in vitro*, and this ability is dependent upon the inclusion of DTT in the assay mix. Activities for both substrates peaked at 0.1-1.0 mM DTT with a slight decrease in activity at 5 mM DTT compared to peak values.

3. *In vivo* assessment of hCPT1 CDP-alcohol specificity

Although it was determined that hCPT1 could use only CDP-choline, and not CDP-ethanolamine, as a substrate under a variety of *in vitro* assay conditions, we sought to confirm this observation *in vivo*. We expressed both hCPT1 and hCEPT1 in yeast strain HJ091 (*cpt1::LEU2 ept1*). The ability of hCPT1 and hCEPT1 to reconstitute the CDP-choline pathway for the synthesis of PC and the CDP-ethanolamine pathway for the synthesis of PE was tested by the addition of radiolabelled choline or ethanolamine, respectively. Expression of hCPT1 resulted in the ability to incorporate radiolabelled choline into PC, but we were unable to detect any radiolabel in the lipid fraction of the ethanolamine-labeled cells (Figure 35). In contrast, expression of hCEPT1 resulted in the reconstitution of both the PC and PE biosynthetic pathways. Hence, the *in vivo* results for

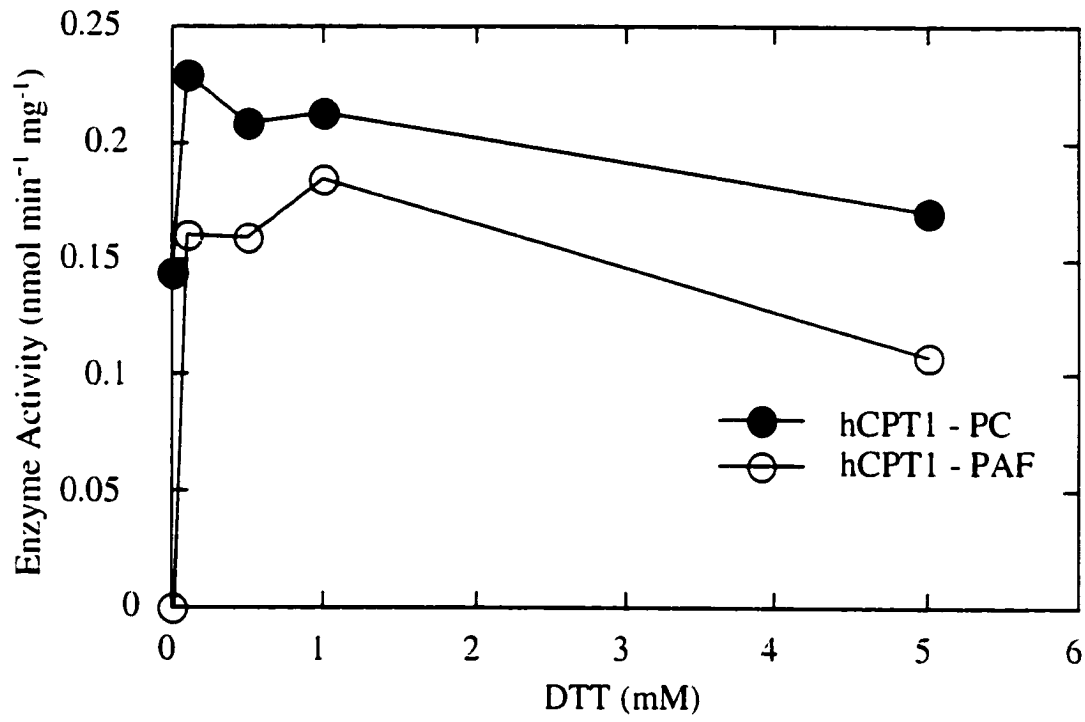


Figure 34. Effect of DTT on hCPT1-derived synthesis of PC and PAF. Enzyme activities were determined from microsomal membranes of HJ091 yeast expressing pAH12 (hCPT1) using the Tween 20 (0.004%) assay as described in Materials and Methods.

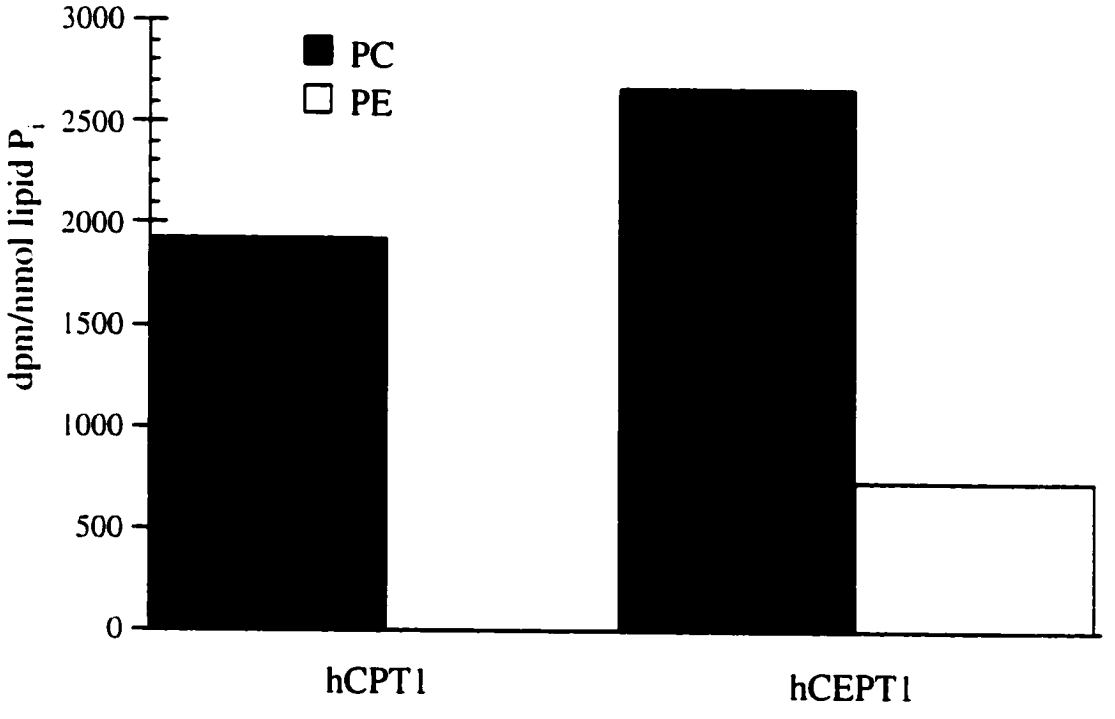


Figure 35. Metabolic labelling of hCPT1 expressed in *S. cerevisiae* HJ091. The hCPT1 or hCEPT1 proteins were expressed in *S. cerevisiae* HJ091 (*cpt1::LEU2 ept1*) and radiolabelled choline or ethanolamine was added to exponential-phase cells for 1 hour as described in Materials and Methods. Lipids were extracted from the cells and the amount of radiolabel incorporated into lipid was determined by scintillation counting.

hCPT1 and hCEPT1 correlated with our *in vitro* assessment of their substrate specificities. It should be noted that although the expression of hCEPT1 was approximately 10-fold higher than hCPT1 in yeast as determined by Western blot and 20-fold higher as assessed by *in vitro* enzyme activities, hCPT1 reconstituted the PC biosynthetic pathway to 70% that of hCEPT1. These rates of PC synthesis were analogous to that observed for the endogenous yeast *CPT1* gene product. The lack of correlation of expression levels with the rate of PC synthesis is consistent with previous observations that the CPT step is normally not rate-limiting for the synthesis of PC. Indeed, either a 5-fold increase, or 10-fold decrease, in yeast *CPT1* activity did not alter the *in vivo* rate of PC synthesis from labeled choline (McMaster and Bell, 1994a).

4. Tissue distribution of hCPT1 mRNA

The relative distribution of hCPT1 mRNA in human tissues was assessed by Northern blot (Figure 36). Due to the small difference in cDNA sequence between hCPT1 and hCPT1β (27-base pair insertion) the blot is likely a composite of the expression of the two splice variants. Only one transcript size was detected, at 1.6 kb. Transcript levels ranged greater than 100-fold between various tissues. The mRNA for hCPT1 was most abundant in testis followed by slightly less transcript in the colon, small intestine, heart, prostate, spleen, and liver. The transcript was also detected in the kidney, skeletal muscle, pancreas, leukocytes, ovary, and thymus. There was very low expression in the brain, placenta, and lung. This is in marked contrast to the transcript levels for human choline/ethanolaminephosphotransferase (hCEPT1), which indicated similar amounts in each of the above tissues.

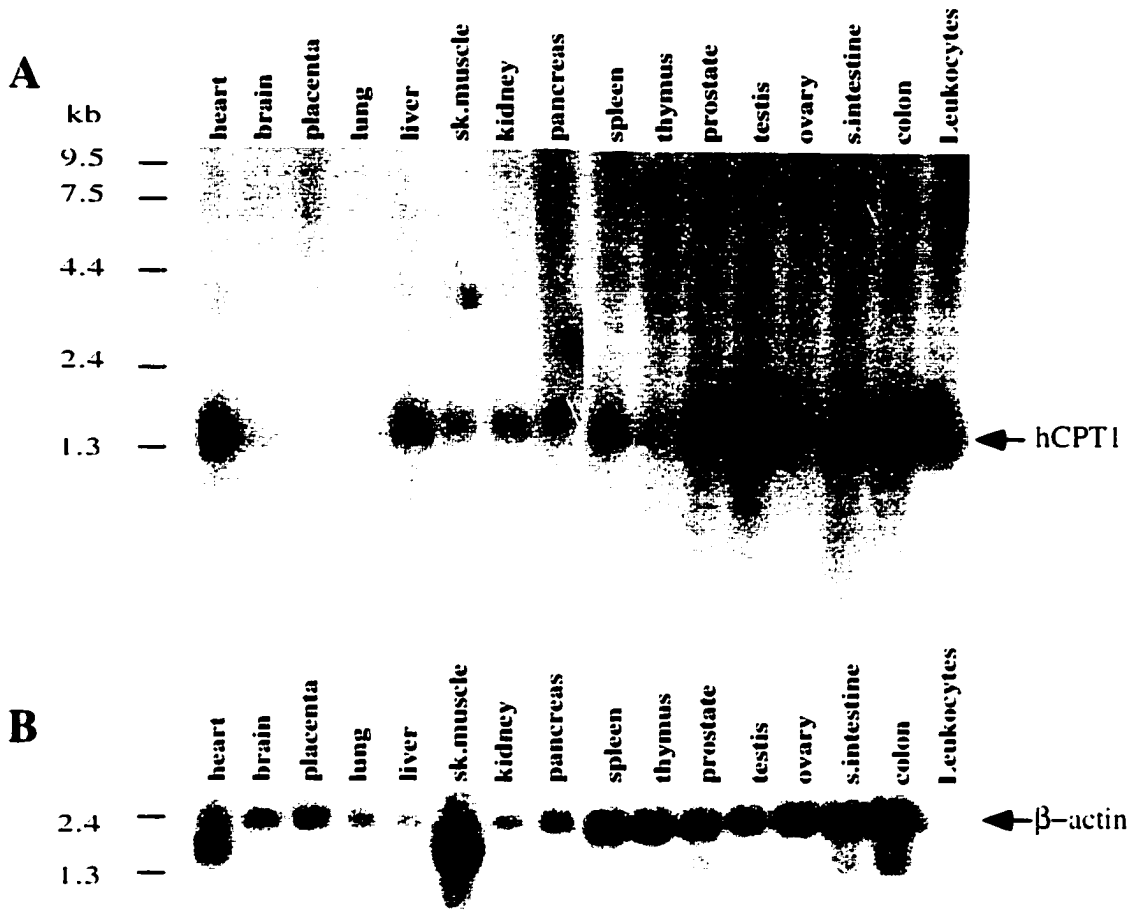


Figure 36. Northern blot analysis of hCPT1 in human cell types. Human multiple tissue mRNA Northern blots were hybridized against probes generated using the random-priming method from (A) the entire 1.2-kb hCPT1 cDNA or (B) a 2.0-kb human β-actin cDNA.

5. Subcellular localization of hCPT1p

To assess the subcellular localization of hCPT1p, we constructed CHO-K1 cell lines that were stably expressing hCPT1 from the plasmid pAH23, which directs the expression of a T7-tagged version of hCPT1 from the CMV promoter. Western blotting with the T7-HRP antibody was used to identify colonies that were stably expressing pAH23. Five out of 30 clones with varying levels of expression were selected for maintenance, and are shown in Figure 37. A band of approximately 39 kDa corresponding to T7-tagged hCPT1p was detected in the membrane-enriched fraction of each cell line. As expected, no band was detected in the cytosolic fraction. A representative of the five clones, hCPT1-8, was used in subsequent indirect immunofluorescence studies.

Due to the lack of antibodies specific for hCPT1p, T7-tagged hCPT1p was detected with a monoclonal antibody to the T7-epitope tag (mT7) in CHO-K1 cells stably expressing pAH23. Cells were visualized by immunofluorescence using a goat anti-mouse secondary antibody that was conjugated to Texas Red. In cells stably transfected with only the vector, pcDNA3, there was a very faint fluorescence in the nucleus when the T7 monoclonal antibody was used (not shown). However, when the mT7 antibody was used to detect T7-hCPT1p in CHO-K1 cells expressing pAH23 (hCPT-8), a fluorescent pattern suggestive of Golgi localization was observed (Figure 38, both panels). To verify that hCPT1p is indeed localized to the Golgi, cells were double-labelled with mT7 and FITC-labelled lentil lectin. Colocalization of T7-hCPT1p and lentil lectin was observed, providing strong evidence that hCPT1p is localized to the Golgi apparatus, unlike hCEPT1p, which was shown to localize in the ER (Figure 20).

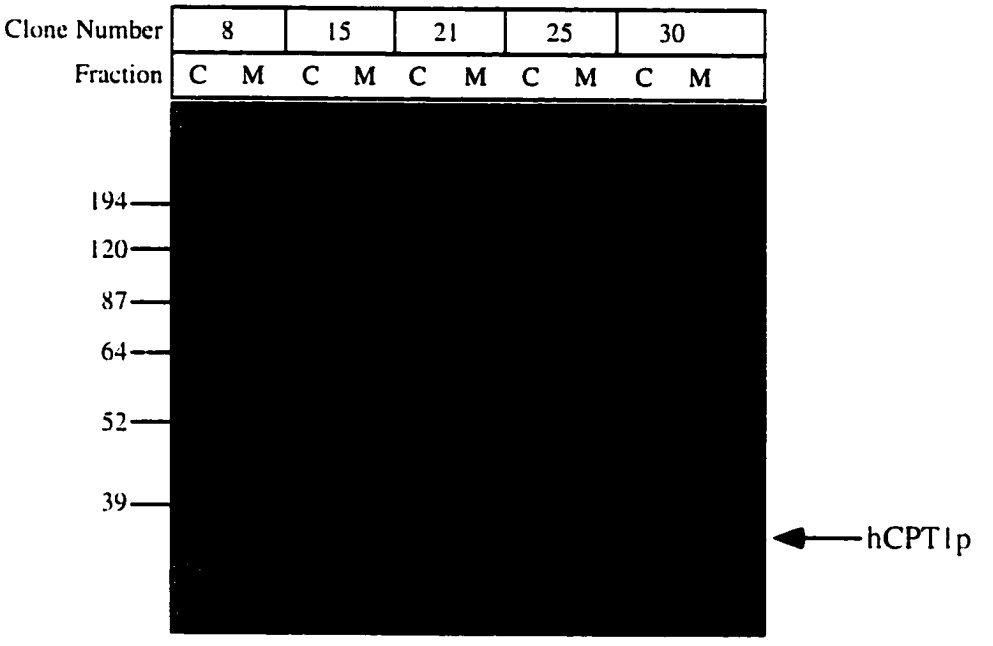


Figure 37. Western blot of CHO-K1 cells stably transfected with hCPT1. CHO-K1 cells were stably transfected with pAH23 using the calcium chloride method as described in Materials and Methods. Subcellular fractions were prepared and 10 μg of either cytosol (C) or membrane (M) fractions were subjected to 10% SDS-PAGE and immunoblotting with T7-HRP (1:5000).

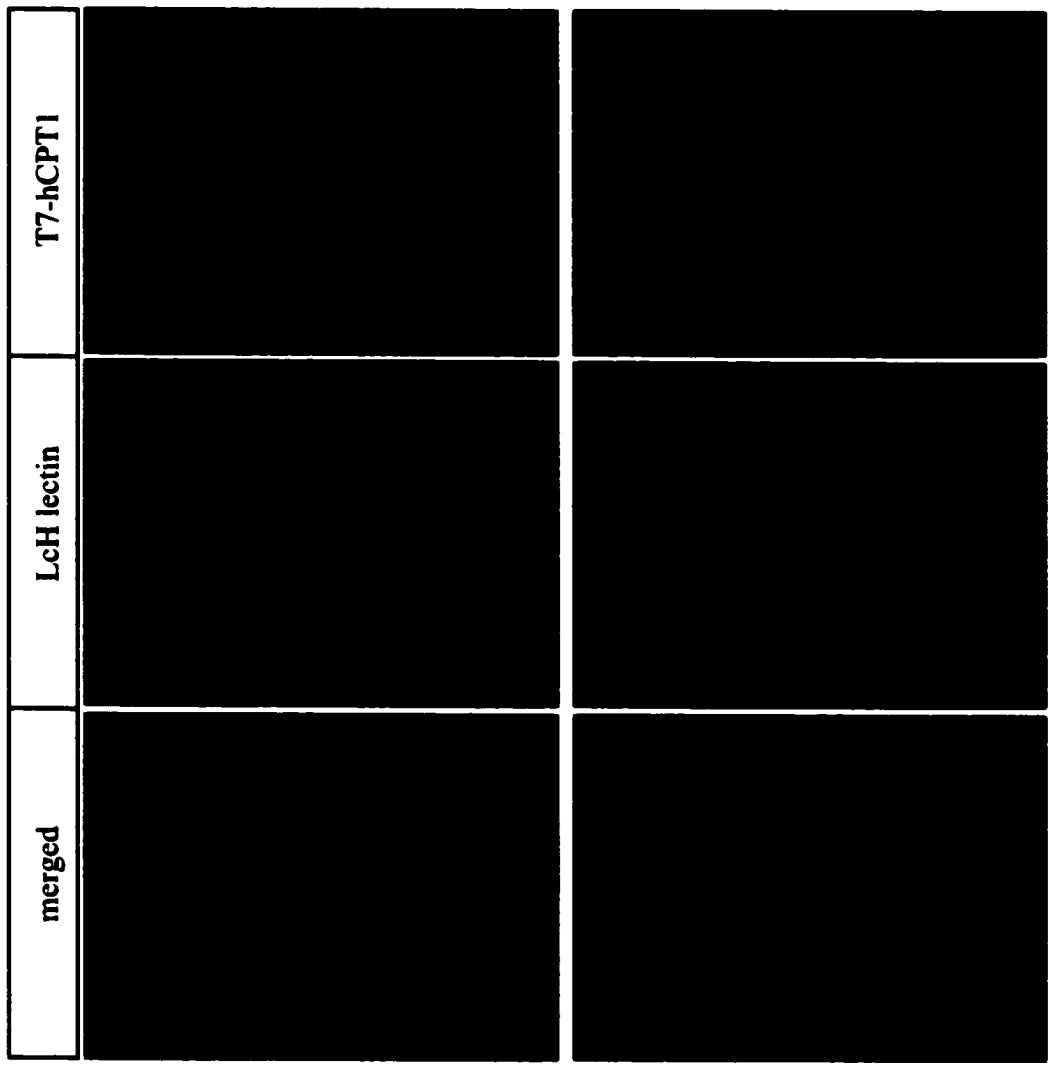


Figure 38. Subcellular localization of hCPT1p. CHO-K1 cells stably transfected with pAH23 (T7-hCPT1) were processed for immunofluorescence using the mT7 antibody (1:1000) to detect hCPT1p and FITC-labelled LcH lectin (1:4000) to detect the Golgi apparatus as described in Materials and Methods. In the merged image, a yellow color indicates colocalization of the two proteins. The left and right panels show two different groups of cells demonstrating similar localizations.

D. Phosphatidylcholine synthesis and *SEC14*-mediated vesicle trafficking

1. Rationale

One of the current hypotheses for the ability of certain gene inactivations to allow survival in the absence of the normally essential *SEC14* gene is that these mutations increase the Golgi DAG pool size (Kearns et al., 1997). Part of the reasoning behind this hypothesis was the observation that inactivation of the yeast *CPT1* gene allowed cell survival in the absence of the normally essential *SEC14* gene product (Cleves et al., 1991). However, inconsistent with the DAG pool size hypothesis was the observation that inactivation of the yeast *EPT1* gene does not allow for bypass of *SEC14* function, even though both the *CPT1* and *EPT1* gene products directly consume DAG for the synthesis of PC and PC/PE, respectively (Cleves et al., 1991). To further explore the relationship between the two Kennedy pathways for the synthesis of PC and PE, and their interaction with *SEC14*-mediated vesicular trafficking events, we instituted an in-depth metabolic, enzymatic, and vesicular transport analysis of each of these pathways.

2. *In vivo* and *in vitro* cholinephosphotransferase activities

We first pulse-labelled yeast containing an inactivated *CPT1* gene (*cpt1::LEU2*), but with an intact *EPT1* gene, with radiolabelled choline at 25°C. This yeast strain, CTY434, synthesized PC at levels 60% of those expressing an intact *CPT1* gene (Figure 39), and overexpression of *EPT1* reconstituted PC biosynthetic levels to 100% those provided by *CPT1*. Disruption of the other two genes coding for enzymes within the Kennedy pathway for PC synthesis, *CKI1* and *PCT1*, reduced PC biosynthesis levels to 5% and 1% of wild-type levels, respectively. Analysis of the metabolites within the Kennedy pathway for PC synthesis were consistent with complete blocks at the choline kinase step

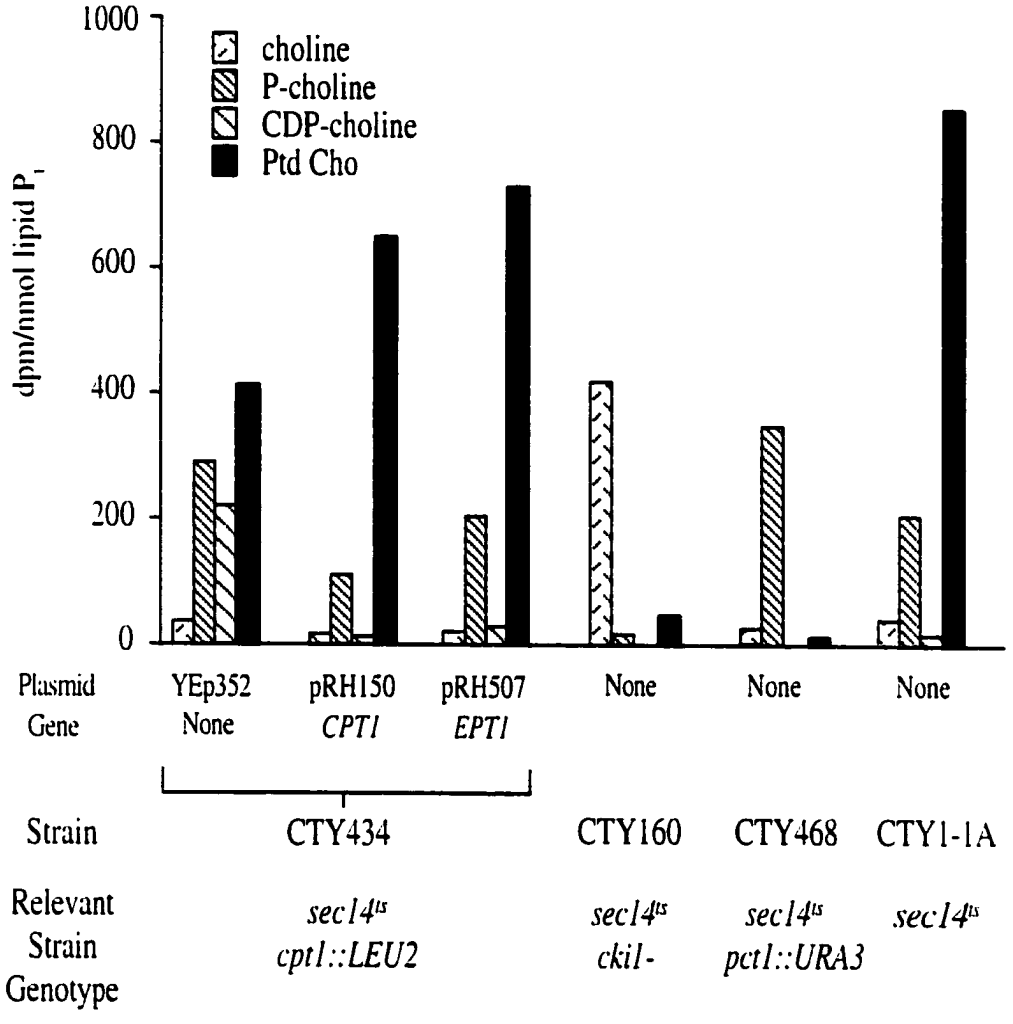


Figure 39. Phosphatidylcholine synthesis *via* the CDP-choline pathway in CTY434 yeast. CTY434 cells (*sec14^{ts} cpt1::LEU2 EPT1*) were transformed with high-copy plasmids containing *CPT1*, *EPT1*, or with YEp352 (vector control). CTY160, CTY468, and CTY1-1A yeast contained only the YEp352 vector. [^{14}C]Choline (10,000 dpm/nmol, 10 μM) was added to 5 ml of mid-log phase yeast cells grown at 25°C. At 1 hour, cells were pelleted by centrifugation and lipids extracted as described in Material and Methods. Phospholipids and CDP-choline pathway metabolites were separated by TLC. Results are the mean of six experiments. SEMs were <15% for each mean.

for yeast containing an inactivated choline kinase gene (*cki1*⁻), an inactivated CTP:phosphocholine cytidylyltransferase gene (*pct1::URA3*), and a partial block in yeast containing an inactivated cholinephosphotransferase gene (*cpt1::LEU2*). These results demonstrate that the partitioning of PC synthesis through the Kennedy pathway enzymes is not mutually exclusive. The Kennedy pathway enzymes could significantly overlap in substrate usage *in vivo* at the ultimate *CPT1*- and *EPT1*-encoded phosphotransferase step.

To ensure the high-copy plasmid-borne *EPT1* and *CPT1* genes were indeed altering CPT activity levels, yeast cells were analyzed using an *in vitro* mixed micelle assay (Table 8) (Hjelmstad and Bell, 1992). Because the yeast cells used in these studies contain an inactivated *CPT1* gene, the endogenous measurable CPT activity was due solely to the remaining *EPT1*-encoded enzyme. Endogenous *EPT1*-encoded CPT activity was determined to be 0.92 nmol min^{-1} mg^{-1} when di18:1 DAG was used as the phosphocholine acceptor substrate, 0.28 nmol min^{-1} mg^{-1} for di16:1 DAG, and no activity was detectable when the short chain di8:0 DAG was provided as the substrate. Overexpression of *EPT1* increased these activities 10-40-fold to 17.42, 9.79, and 0.17 nmol min^{-1} mg^{-1} for di18:1, di16:1, and di8:0 DAGs, respectively. As expected, overexpression of *CPT1* also increased CPT activity to 13.01 nmol min^{-1} mg^{-1} for di16:1 DAG, versus 4.11 nmol min^{-1} mg^{-1} for di18:1 DAG and 0.20 nmol min^{-1} mg^{-1} for di8:0 DAG.

3. Phosphatidylcholine synthesis and vesicle trafficking

The study of the essential *SEC14*-encoded PC/PI transfer protein has been facilitated by the isolation of a conditional temperature-sensitive *SEC14* allele, $sec14^{ts}$ (Cleves et al., 1991). At the permissive temperature of 25°C, cells containing the $sec14^{ts}$ allele possess

Table 8. Cholinephosphotransferase activity of *S. cerevisiae* strain CTY434

Diacylglycerol substrate	Plasmid/Gene	CPT Activity (nmol min^{-1} mg^{-1})
di16:1	YEp352/none	0.28 ± 0.02
	pRH150/CPT1	13.01 ± 0.51
	pRH507/EPT1	9.79 ± 0.45
di18:1	YEp352/none	0.92 ± 0.16
	pRH150/CPT1	4.11 ± 0.22
	pRH507/EPT1	17.42 ± 0.74
di8:0	YEp352/none	undetectable
	pRH150/CPT1	0.20 ± 0.06
	pRH507/EPT1	0.17 ± 0.01

Cholinephosphotransferase activities were determined in microsomal preparations from *S. cerevisiae* CTY434 cells (*sec14*^{ts} *cpt1::LEU2 EPT1*) harboring either Yep352 (vector control), pRH150 (*CPT1*), or pRH507 (*EPT1*), using the Triton X-100 mixed micelle assay as described in Materials and Methods. Results are shown the average ± SEM for at least four experiments.

normal *SEC14*-mediated PC/PI transfer activity and thus can transport vesicles from the Golgi and grow normally. Upon raising the cell culture conditions to 37°C, a nonpermissive temperature for the *sec14*^{ts} allele, cells can no longer catalyze the PC/PI transfer *in vitro*, and *in vivo* cells cease Golgi-mediated vesicular transport and eventually die (Bankaitis et al., 1990; Cleves et al., 1991). It has been previously observed that mutations within the *CPT1* gene bypass the requirement for a functional *SEC14* gene product and allow *sec14*^{ts} cells to grow at 37°C, but mutations in the *EPT1* gene do not restore secretory competence or cell viability.

Previous *in vivo* metabolic analysis of the partitioning of the Kennedy pathways revealed that *CPT1*-encoded activity synthesized the majority of PC and barely detectable levels of PE. Conversely, *EPT1*-encoded activity synthesized a small amount of PC and the bulk of Kennedy pathway-derived PE (McMaster and Bell, 1994a). In addition, increased expression of *EPT1* did not affect its contribution toward the synthesis of PC. In the current study, we were surprised by our observation that in the yeast strain CTY434, which contained an inactivated *CPT1* gene but an intact *EPT1* gene, the level of PC synthesis was 60% that of wild-type, and overexpression of *EPT1* restored PC synthesis to 100% wild-type levels (Figure 39). These data imply that there are as yet uncharacterized cellular events that can alter the ability of *EPT1* to significantly contribute to the synthesis of PC. However, these same observations now provided an experimental system to examine how alterations in consumption of endogenous DAG by yeast enzymes affect *SEC14*-mediated vesicular transport processes and cell viability.

The yeast strain that contains the active *EPT1* gene and inactivated *CPT1* gene used in the metabolic labelling experiments described above (CTY434) also contains a

conditional temperature-sensitive *sec14*^{ts} allele. Normally, an inactivated *CPT1* gene (*cpt1::LEU2*) would be predicted to allow a bypass of the essential requirement for *SEC14* and allow the yeast to survive and successfully transport vesicles from the Golgi apparatus at both the *sec14*^{ts}-permissive temperature of 25°C and the *sec14*^{ts}-nonpermissive temperature of 37°C. We tested whether disruption of the *CPT1* gene in the CTY434 strain, which resulted in the surprisingly high level of PC synthesis due to the remaining *EPT1* gene, was still capable of bypassing the cellular requirement for a functional *SEC14* gene product (Figure 40A). The 40% decrease in Kennedy pathway-derived PC synthesis in the CTY434 yeast strain was sufficient to allow survival in the face of a nonfunctioning *SEC14* gene product. Upon overexpression of *EPT1* or *CPT1*, PC synthesis was restored to 100% wild-type levels, and these yeast cells could no longer survive at 37°C, indicating the reimposition of the requirement for a functional *SEC14* gene product.

To correlate the observed effects on cell growth with alterations in vesicular transport, the ability of each of the strains to secrete invertase was measured after cells were shifted to the *sec14*^{ts}-nonpermissive temperature of 37°C (Figure 41). Cells containing the *sec14*^{ts} mutation alone displayed a significant decrease in the ability to secrete invertase compared with wild-type yeast. In cells containing the *sec14*^{ts} allele, inactivation of the first two enzymes of the Kennedy pathway for the synthesis of PC, choline kinase (*CKI1*) and CTP:phosphocholine cytidylyltransferase (*PCT1*), resulted in an increase in invertase secretion to levels at or near those of wild-type cells. Interestingly, the ability to secrete invertase was only moderately increased in *sec14*^{ts} cells containing an inactivated *CPT1* gene. Overexpression of *EPT1* or *CPT1* in CTY434

Figure 40. *EPT1* and di8:0 diacylglycerol affect *SEC14*-mediated cell growth. (A) CTY434 yeast (*sec14*^{ts} *cpt1::LEU2 EPT1*) transformed with *CPT1* or *EPT1* in the high-copy vector YEp352, or YPP649.7 (*sec7*^{ts}), YPP649.13 (*sec13*^{ts}), and YPP649.15 (*sec15*^{ts}) transformed with YEp352, were grown overnight at 25°C in appropriate selective medium to ensure plasmid maintenance and diluted to identical cell number as determined spectrophotometrically by culture absorbance at 600 nm. Serial dilutions (1:10) were performed, and 1 µl of each dilution was plated onto SC minus uracil solid medium. (B) The identical experiment as in panel A, except 1 µl of each dilution was plated onto SC minus uracil solid medium containing 200 µM di8:0 DAG. All plates were incubated for 4 days at 25°C or 7 days at 37°C. [*CPT1*] and [*EPT1*] indicated the presence of YEp352 plasmid-borne genes.

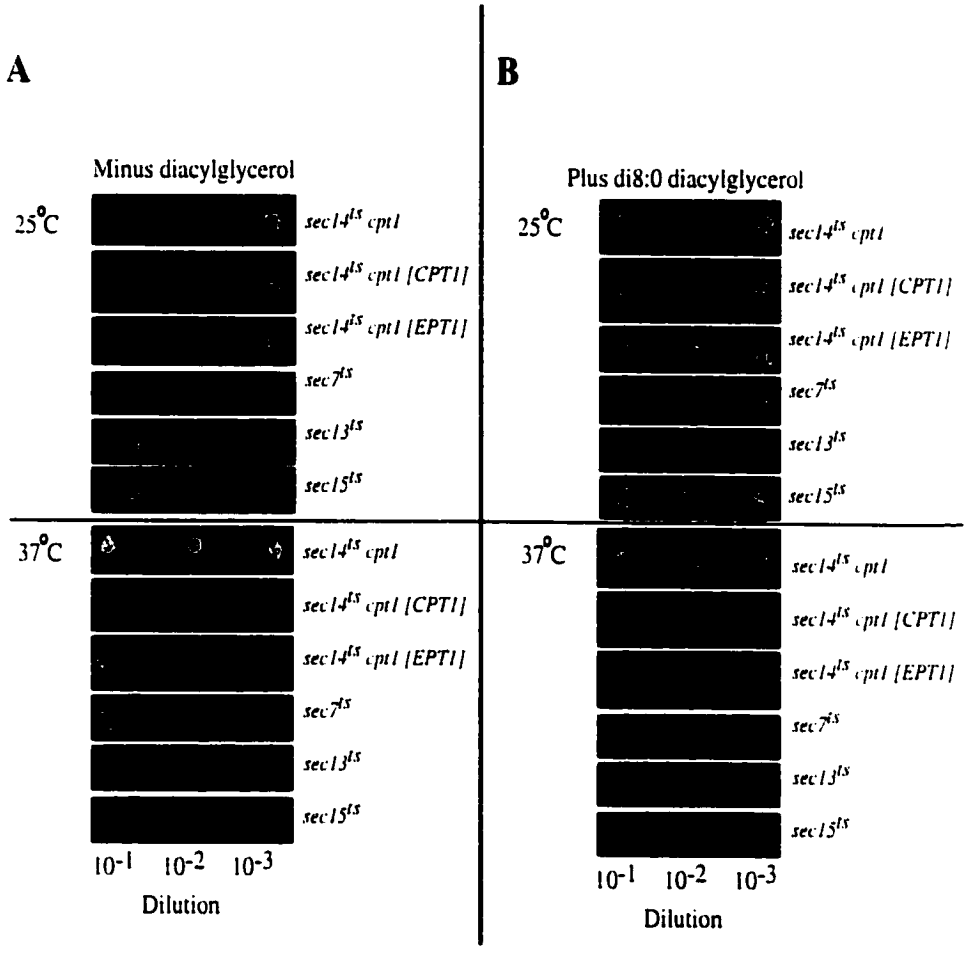

A
B
Minus diacylglycerol
Plus di8:0 diacylglycerol
25°C
37°C
sec14ts cpt1
sec14ts cpt1 [CPT1]
sec14ts cpt1 [EPT1]
sec7ts
sec13ts
sec15ts
10-1 10-2 10-3
Dilution

Figure 40

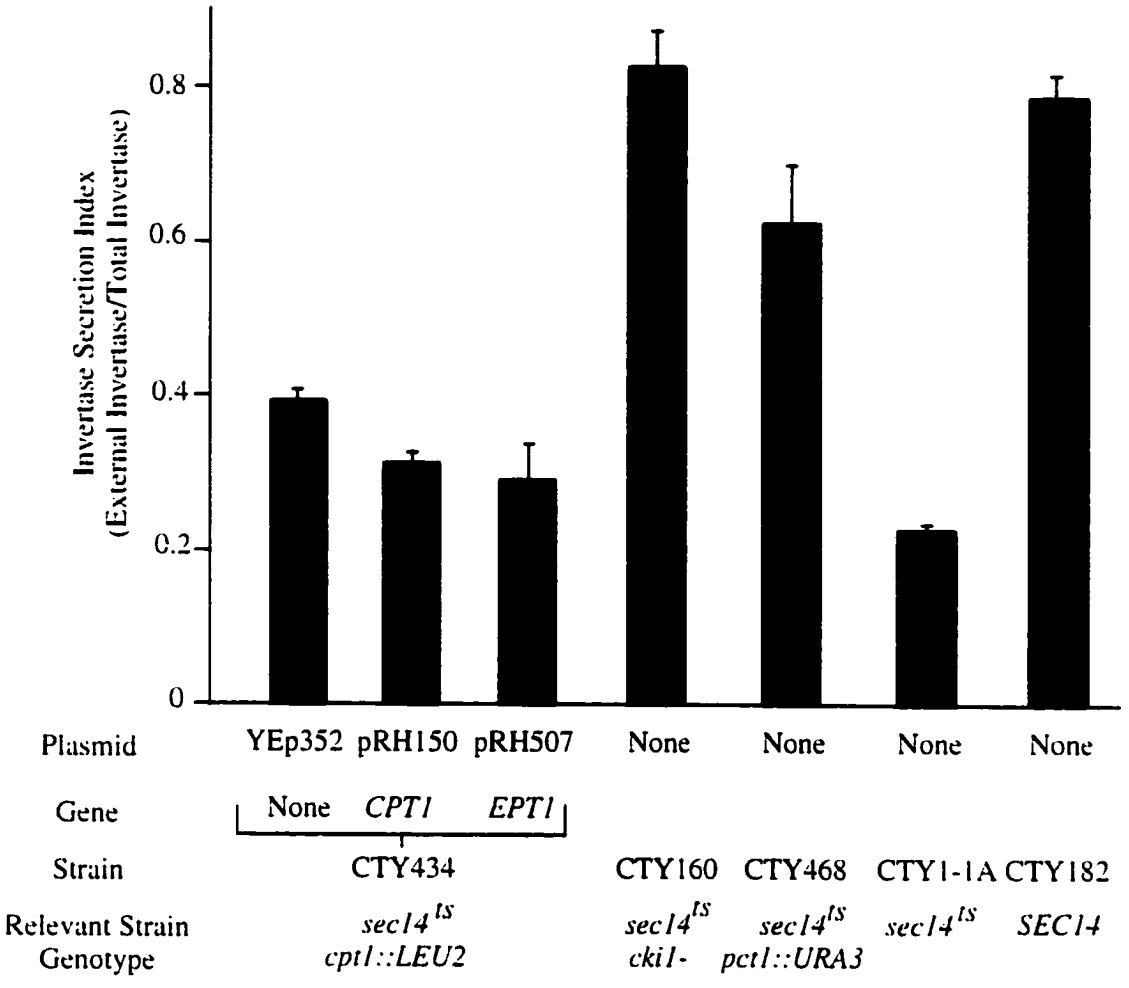


Figure 41. Invertase secretion indices. Yeast strains CTY182 (wild type, *SEC14 CPT1 EPT1*), CTY1-1A (*sec14*^{ts} *CPT1 EPT1*), CTY160 (*sec14*^{ts} *ckl1*), CTY468 (*sec14*^{ts} *pct1::URA3*), or CTY434 (*sec14*^{ts} *cpt1::LEU2 EPT1*) transformed with the vector control YEp352, or with *CPT1* or *EPT1* in the high-copy vector YEp352, were grown to mid-log phase in YEPD (2% glucose) medium at 25°C. Cells were pelleted (2000 x *g* for 1 min), washed twice with water, and resuspended in 5 ml YPD containing 0.1% glucose. Cultures were subsequently grown at 37°C to impose the temperature-sensitive phenotype and invertase activities determined as described in Materials and Methods. Results are shown as the mean of six experiments ± SEM, except for CTY160 and CTY468, in which n = 3.

cells decreased invertase secretion to near those observed for cells containing the *sec14*^{ts} allele alone. Total invertase enzyme activities were not affected by ablation of Sec14p function compared with wild-type yeast, and the restoration of secretion competency through inactivation of the genes of the CDP-choline pathway did not significantly alter total invertase enzyme activity. Compared with wild-type yeast (100%), the *sec14*^{ts} strain possessed total invertase activity of 96% wild-type, whereas the bypass suppressors, containing both the *sec14*^{ts} mutation and disruptions of each of the genes of the CDP-choline pathway, had invertase activities ranging from 65% to 103% those of wild-type yeast.

To sum, complete abolition of PC synthesis in cells grown at the nonpermissive temperature for the *sec14*^{ts} allele resulted in invertase secretion at wild-type levels (3-4-fold above those observed in cells containing the *sec14*^{ts} allele alone). However, PC synthesis at 60% wild-type levels increased invertase secretion only 1.5-2-fold (compared with the *sec14*^{ts} allele alone), but was sufficient to allow life, whereas restoration of PC synthesis to 100% wild-type levels reimposed the requirement for functional *SEC14* on the cells. Hence, the rate of invertase secretion correlated directly with the rate to which PC synthesis was decreased. These results are consistent with the hypothesis that the rate of endogenous DAG consumption directly affects the requirement for *SEC14*. However, lipid pathways downstream of DAG consumption or PC synthesis may also impact on *SEC14*-mediated events.

4. Effect of di8:0 diacylglycerol on cell growth and lipid metabolism

Di8:0 DAG is a synthetic lipid not produced in eukaryotic cells, but its increased solubility compared with long-chain DAGs has been exploited to allow its use as a

pharmacological tool to demonstrate DAG-specific regulatory and signalling events (Davis et al., 1985; Kearns et al., 1997). Di8:0 DAG was added to the medium of CTY434 (*sec14*^{ts} *cpt1::LEU2*) yeast cells, which contained plasmid-borne *EPT1* or *CPT1* genes, or p416 GPD (vector control). The addition of di8:0 DAG rescued cells *via* a reproducible partial suppression of the *sec14*^{ts} growth phenotype at 37°C and this was independent of whether cells were overexpressing *CPT1* or *EPT1* (Figure 40B). The addition of di8:0 DAG did not rescue growth at the nonpermissive temperature for *sec7*^{ts} and *sec13*^{ts} yeast, which are defective in endoplasmic reticulum to Golgi transport, and *sec15*^{ts} cells, which are defective in secretion at the Golgi to plasma membrane step. These results imply that the rescue of cell growth by di8:0 DAG is specific to Sec14p-mediated events. In addition, the lack of correlation between *CPT1* and *EPT1* expression and the ability of di8:0 DAG to alter *SEC14*-mediated secretion is consistent with our *in vitro* observation that di8:0 DAG was a poor substrate for these enzymes (Table 8). The above mentioned data indicate that di8:0 DAG is likely not metabolized by either *CPT1*- or *EPT1*-derived activities. However, to effectively examine the role of DAG on *SEC14*-mediated vesicular transport events, the effect of di8:0 DAG on DAG pool sizes and lipid metabolism parameters was measured.

The addition of 200 μM di8:0 DAG to early log-phase yeast resulted in an accumulation of di8:0 DAG to 20-40 times those of endogenous long-chain DAG levels (Figure 42). Overexpression of *CPT1* or *EPT1* did not affect the levels of di8:0 DAG, indicating that these enzymes were not effectively consuming di8:0 DAG, consistent with the *in vitro* substrate specificity data and the di8:0 DAG cell growth assays (Table 8 and Figure 40). Although the combined results indicate that di8:0 DAG is not effectively

Figure 42. Effect of exogenous di8:0 diacylglycerol administration on diacylglycerol pool sizes. CTY434 cells ± plasmids were grown overnight at 25°C in synthetic dextrose medium containing the appropriate nutrients. Optical densities were measured at 600 nm and each strain was diluted to A_{600} of 0.150. Cells were grown for 1 hour at 25°C and the medium was subsequently supplemented with di8:0 DAG to a final concentration of 200 μM. Five-milliliter samples were removed at the indicated time points and diacylglycerols were extracted and quantified as described in Materials and Methods. (A) CTY434 cells grown without di8:0 DAG supplementation. (B) CTY434 cells grown with di8:0 DAG supplemented to 200 μM. (C) CTY434 cells containing high-copy plasmids carrying the *CPT1* or *EPT1* genes supplemented to 200 μM di8:0 DAG for 4 hours. Results are the mean of four experiments. SEMs were <15% for each mean.

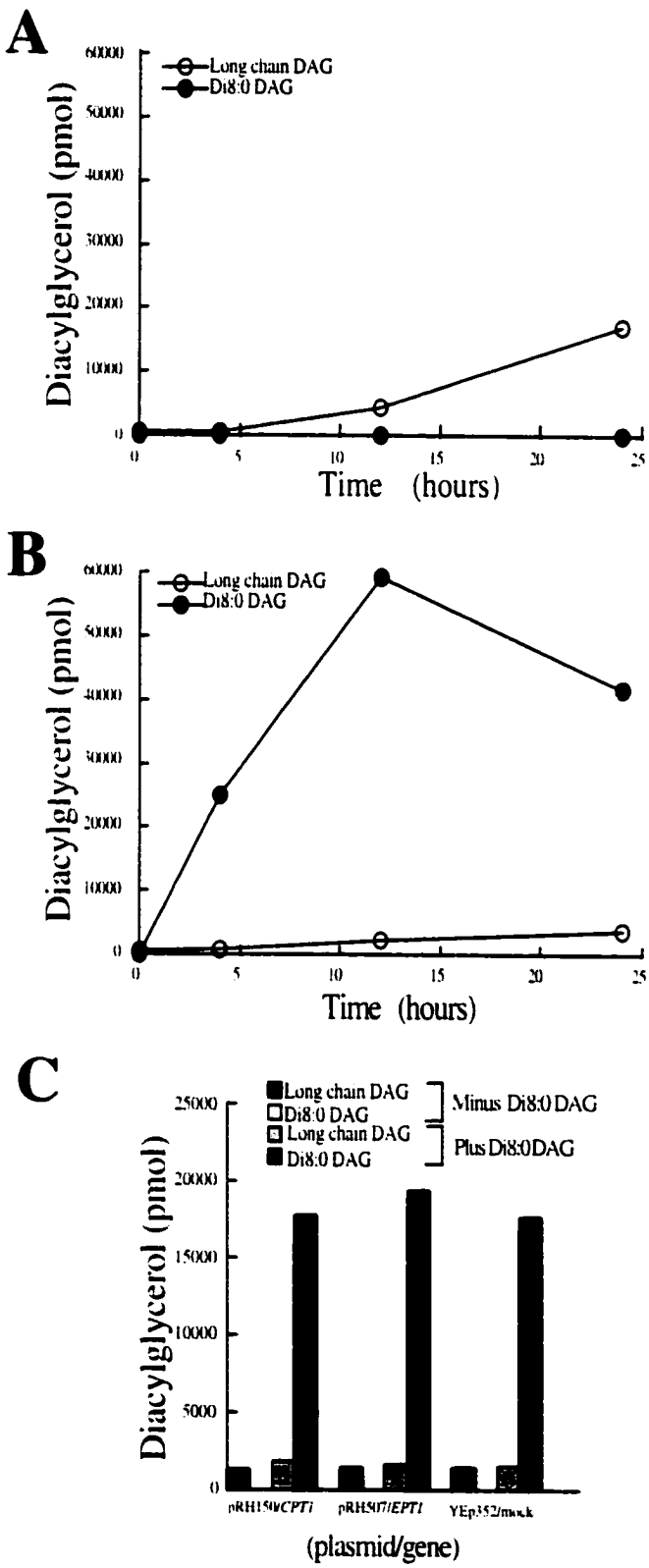

A
Long chain DAG
Di8:0 DAG
Diacylglycerol (pmol)
Time (hours)
B
Long chain DAG
Di8:0 DAG
Diacylglycerol (pmol)
Time (hours)
C
Long chain DAG
Di8:0 DAG
Minus Di8:0 DAG
Long chain DAG
Di8:0 DAG
Plus Di8:0DAG
Diacylglycerol (pmol)
(plasmid/gene)

Figure 42

metabolized by yeast, and almost certainly not via *CPT1*- or *EPT1*-derived activities, the vast increase in intracellular di8:0 DAG levels prompted us to examine whether di8:0 DAG administration affected gross lipid metabolism. The incorporation of phosphorus-32 into CTY434 yeast in the presence or absence of 200 μM di8:0 DAG was determined. Total net lipid synthesis was not affected by the addition of di8:0 DAG (Figure 43). Phospholipids within the lipid fraction were separated by two-dimensional TLC and the plates were exposed to x-ray film. The addition of di8:0 DAG did not affect the incorporation of phosphorus-32 into PC, PE, PI, PS, PA, CDP-DAG, phosphatidylglycerol, or cardiolipin. Importantly, we also noted that there was no conversion of the di8:0 DAG to di8:0 PA because there was no radiolabel associated with the mobility of this lipid in the two-dimensional TLC system used. We demonstrated that the lipid extraction protocol used in this study efficiently extracted the majority of di8:0 PA. In addition, a visual analysis of the x-ray film did not reveal any uniquely labelled regions upon the addition of di8:0 DAG. Hence, the data are consistent with di8:0 DAG entering cells and dramatically increasing cellular DAG levels. However, di8:0 DAG did not appear to alter phospholipid metabolic pathways, or to be converted to di8:0 PA.

5. Role of hCPT1 and hCEPT1 in *SEC14*-mediated vesicle trafficking

As a preliminarily test whether hCPT1 or hCEPT1 might be involved in the regulation of vesicle trafficking, these enzymes were expressed in a yeast strain containing both a *sec14*^{ts} temperature-sensitive allele and an inactivated yeast *CPT1* gene (CTY434, *sec14*^{ts} *cpt1::LEU2*). Since both hCPT1 and hCEPT1 could reconstitute PC synthesis in yeast lacking endogenous CPT activity (although hCPT1 synthesized PC at 30% that of hCEPT1), we tested whether hCPT1 or hCEPT1 could also complement

Figure 43. Effect of exogenous di8:0 diacylglycerol administration on phospholipid synthesis. Inorganic phosphorus-32 (2.5 mCi) was added to 20 ml of mid-log-phase CTY434 cells grown at 25°C in yeast minimal medium containing appropriate nutritional supplements. At 4, 12, and 24 hours, a 5-ml sample was removed and centrifuged at 3000 x *g* for 5 minutes to pellet cells. Cells were washed twice with 5 ml of ice-cold water and lipids were extracted and quantified as described in Material and Methods. (A) Incorporation of phosphorus-32 into total phospholipid. (B) Incorporation of phosphorus-32 into individual lipid classes. (C) Incorporation of phosphorus-32 into individual lipid classes in the presence of 200 μM di8:0 DAG. Results are the mean of four experiments. SEMs were <15% for each mean.

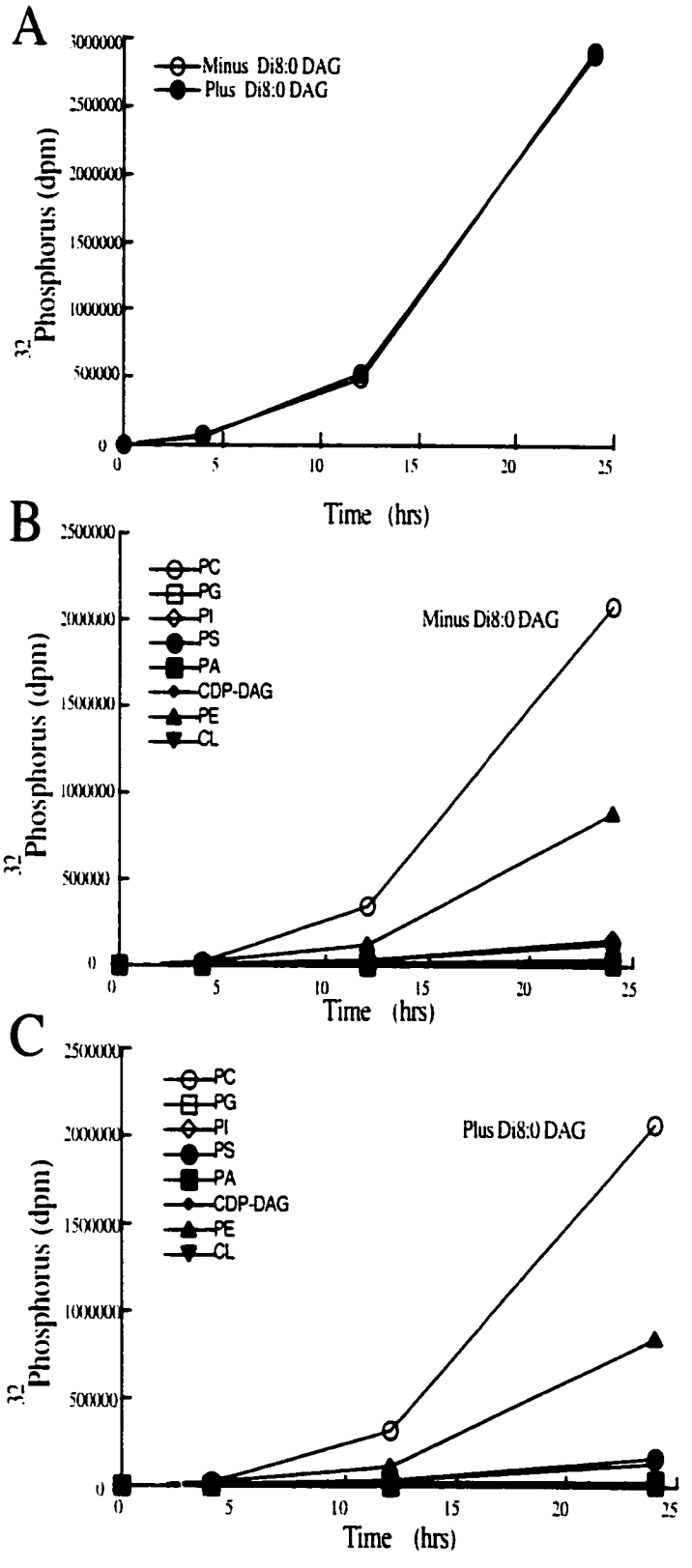

A
Minus Di8:0 DAG
Plus Di8:0 DAG
32 Phosphorus (dpm)
Time (hrs)
B
PC
PG
PI
PS
PA
CDP-DAG
PE
CL
Minus Di8:0 DAG
32 Phosphorus (dpm)
Time (hrs)
C
PC
PG
PI
PS
PA
CDP-DAG
PE
CL
Plus Di8:0 DAG
32 Phosphorus (dpm)
Time (hrs)

Figure 43

yeast *CPT1* function with respect to *SEC14*-mediated Golgi vesicular transport. Either hCPT1, hCEPT1, or yeast *CPT1* was expressed in CTY434 (*sec14*^{ts} *cpt1::LEU2*) and the ability of the cells to grow at the permissive (25°C) and non-permissive (37°C) *sec14*^{ts} temperature was determined. As expected, expression of the yeast *CPT1* enzyme resulted in cells that were unable to grow at 37°C due to restoration of the *sec14*^{ts} phenotype (Figure 44). Expression of hCEPT1p in CTY434 yeast also resulted in cells unable to grow at 37°C, indicating hCEPT1 activity can successfully complement the yeast *CPT1*-derived protein with respect to its interaction with *SEC14*-mediated vesicular transport events. Surprisingly, expression of hCPT1 in the same yeast did not affect growth at the non-permissive temperature of 37°C. Hence, although expression of hCEPT1 or hCPT1 each reconstituted PC synthesis to levels similar to those provided by the endogenous yeast *CPT1* gene, hCEPT1, but not hCPT1, inhibited cell survival in the absence of a functional Sec14 protein.

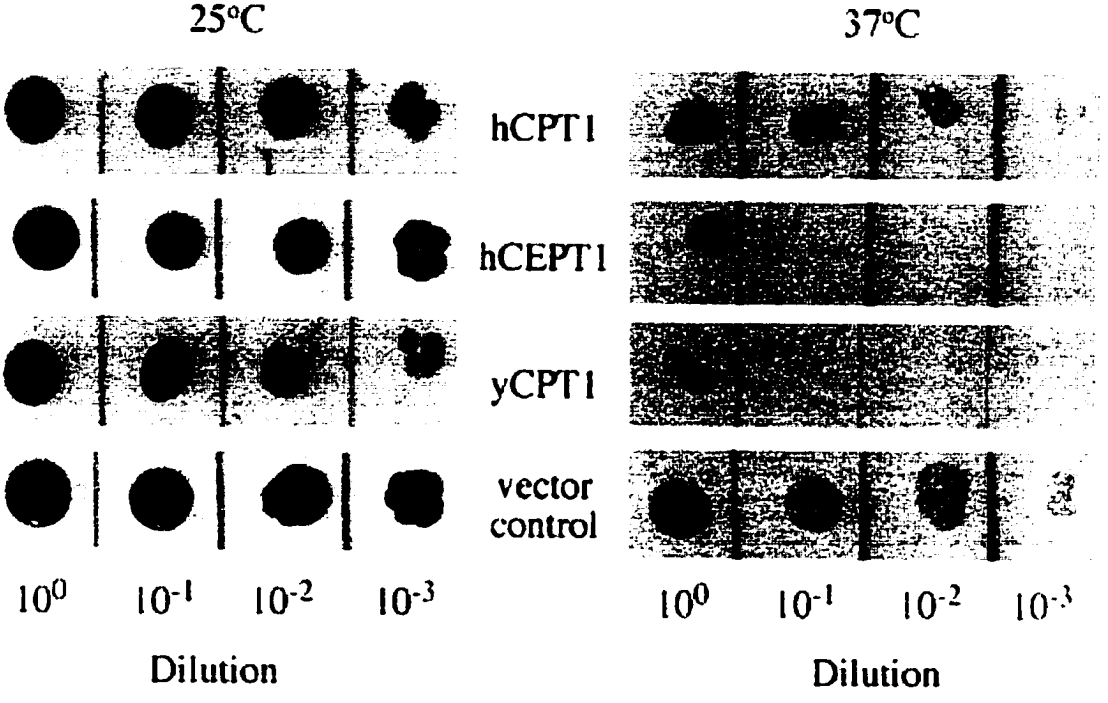


Figure 44. Ability of hCPT1 and hCEPT1 to affect *SEC14*-dependent cell growth. *S. cerevisiae* CTY434 cells containing a temperature-sensitive *SEC14* allele (*sec14*^{ts}) and an inactivated *CPT1* gene (*cpt1::LEU2*) were transformed with plasmids for the constitutive expression of hCPT1, hCEPT1, and yeast *CPT1*, and also with an empty vector control. Cells were grown overnight in liquid medium at 25°C and identical numbers of cells were serially diluted 1:10 and spotted onto minimal solid medium containing the required nutrients for plasmid maintenance. Plates were incubated at 25°C or 37°C for 4 days.

V. Discussion

A. The human complement of choline/ethanolaminephosphotransferases

The final steps in the Kennedy pathways for the synthesis of PC and PE are catalyzed by cholinephosphotransferase and ethanolaminephosphotransferase activities, respectively. This study describes the first isolation and expression of mammalian cDNAs, hCEPT1 and hCPT1, coding for either a cholinephosphotransferase or an ethanolaminephosphotransferase activity. The cloned hCEPT1 described in our study codes for a dual specificity choline/ethanolaminephosphotransferase capable of utilizing both CDP-choline and CDP-ethanolamine as phosphobase donors to a broad range of diacylglycerols, resulting in the synthesis of both PC and PE (Table 3). *In vitro* kinetic analysis revealed that CDP-choline was preferred as the phosphobase donor in the presence of Mg^{2+}, while CDP-ethanolamine was preferred in the presence of Mn^{2+} (Figure 13). Both the CPT and EPT activities of hCEPT1p possessed an absolute requirement for either Mg^{2+} or Mn^{2+}. This same cation preference was also observed for a partially purified choline/ethanolaminephosphotransferase activity from rat liver (Kanoh and Ohno, 1976). Two mechanisms have been proposed by which cations activate CPTs and EPTs. Either cations bind to the CDP-aminoalcohol substrate or there exists a defined cation-binding site within the enzyme (McMaster and Bell, 1997; McMaster et al., 1996); however, resolution of the mechanism of cation activation and also of the cation used *in vivo* awaits protein purification. To test whether the ability of hCEPT1p to utilize both CDP-choline and CDP-ethanolamine as substrates could be repeated *in vivo*, hCEPT1 was expressed in an *S. cerevisiae* strain devoid of endogenous CPT and EPT activity (HJ091, *cpt1::LEU2 ept1*) and radiolabelled choline or ethanolamine was added to

exponential-phase cells in an attempt to reconstitute each pathway. Both the CDP-choline and CDP-ethanolamine pathways could be reconstituted by hCEPT1 expression (Figure 15), indicating that hCEPT1 has the ability to synthesize both PC and PE *in vitro* and *in vivo*.

The hCPT1 cDNA cloned and described in this study encodes an enzyme that demonstrated cholinephosphotransferase activity in *in vitro* enzyme assays (Table 7). When hCPT1 was expressed in *S. cerevisiae* HJ091 (*cpt1::leu2 ept1*), it was also able to reconstitute the PC biosynthetic pathway *in vivo* (Figure 35). However, in contrast to hCEPT1, the hCPT1 enzyme was unable to use CDP-ethanolamine as a substrate *in vitro* and was unable to reconstitute PE biosynthesis *in vivo*. In the context of mammalian cell phospholipid synthesis, it has been presumed that separate CPT and EPT activities exist (Kent, 1995; Vance, 1996), especially in light of the isolation of the CHO cell line with decreased EPT but normal CPT activity (Polokoff et al., 1981). However, the redundancy of CPT activity in both the hCEPT1 and hCPT1 enzymes, and the dual specificity of hCEPT1 for the synthesis of PC and PE, imply that these two pathways may not be metabolically distinct. The specific deficiency in ethanolaminephosphotransferase activity for this CHO cell line could be explained in several ways: (1) an as yet unidentified structural gene distinct for EPT activity exists; (2) a gene capable of utilizing both CDP-choline and CDP-ethanolamine as substrates was mutated such that CDP-ethanolamine was now used less effectively as a substrate; (3) a gene that codes for an activity that regulates the partitioning of phospholipid biosynthetic pathways was mutated such that the synthesis of PC was favored over that of PE; or (4) hCEPT1 activity was inactivated, while hCPT1 activity remained. Unfortunately, the expression of hCEPT1 in

this cell line cannot be performed because this line is no longer available. Whether the supply of specific diacylglycerols and CDP-aminoalcohols affects the ability of hCEPT1p to partition the PC and PE biosynthetic pathways deserves further characterization. Consistent with this notion of substrate supply affecting CPT or EPT activity *in vivo*, diacylglycerol levels have previously been demonstrated to limit the synthesis of both PC (Jamil et al., 1992) and PE (Tijburg et al., 1989) at the level of CPT and EPT activities, respectively.

A PAF-specific CPT activity had been previously distinguished from the PC-specific CPT enzyme based on DTT sensitivity (Snyder, 1997; Woodard et al., 1987). Microsomal membranes contained a PAF-specific CPT activity that was moderately stimulated by DTT and a PC-specific CPT activity that was inhibited by DTT. We found that the cloned hCPT1 and hCEPT1 were both able to synthesize PAF *in vitro*. The hCPT1 PAF-synthesizing activity was stimulated by and dependent on DTT, while the hCEPT1 PAF-synthesizing activity was unaffected by DTT. Neither hCPT1 nor hCEPT1 PC-biosynthetic activity was inhibited by DTT. Hence, neither the hCPT1- nor the hCEPT1-encoded CPT activities reflect those previously observed in mammalian microsomes for the synthesis of PAF and PC. As yeast do not contain ether-linked lipids, the precise *in vivo* substrate specificity for the synthesis of ether-linked PC molecules by hCPT1 and hCEPT1 awaits the development of mammalian systems for subsequent analyses. However, simple overexpression systems are unlikely to provide insight into hCPT1 and hCEPT1 substrate specificities as the cholinephosphotransferase step is not rate-limiting in the pathway, and thus more complicated knockout or antisense strategies will likely have to evolve and these are currently under development.

The hCEPT1 transcript was detected in all cell types tested and was not enriched in any particular tissue (Figure 16). In contrast, the hCPT1 mRNA levels varied greater than 100-fold among the same tissues, implying a tissue-specific expression of hCPT1 (Figure 36). The ubiquitous nature of the hCEPT1 mRNA is consistent with the ability of hCEPT1 to synthesize PC and PE *in vivo*. To date, hCEPT1-directed synthesis of PE is the only known way in which cells can make PE *de novo*. Therefore, it would be predicted that hCEPT1 would be expressed in all cells.

Colocalization of hCEPT1p with organelle-specific markers revealed that it is localized to the ER and nuclear membrane (Figures 20 and 21), consistent with a number of previous studies analyzing CPT activity in subcellular fractions. However, CPT activity has also been reported in the Golgi apparatus (Jelsema and Morre, 1978; Vance and Vance, 1988) and mitochondria (Schlame et al., 1989; Sikpi and Das, 1987), and our results demonstrate that hCEPT1p is not found associated with either of these organelles. Interestingly, in addition to its ER localization, hCEPT1p is found in the nuclear membrane, as demonstrated by its ability to colocalize with CTα in the presence of oleic acid. When activated, CTα translocates to the nuclear membrane to catalyze the conversion of phosphocholine to CDP-choline, the step immediately preceding CPT in the *de novo* biosynthesis of PC. Therefore, it is tempting to speculate that hCEPT1p and CTα colocalize at the nuclear membrane to facilitate the movement of the water-soluble CDP-choline substrate. It would be interesting to determine whether hCEPT1p and CTα actually interact physically. In contrast to hCEPT1p, hCPT1p was localized to the Golgi apparatus, as shown by its colocalization with LcH-lectin (Figure 38). This is a

significant finding in light of the fact CPT activity has been found in both the ER and the Golgi.

Analysis of the amino acid sequences of both hCEPT1p and hCPT1p revealed 6-8 predicted membrane-spanning helices, depending on the algorithm used. Based on the previous predictions for the *S. cerevisiae CPT1* and *EPT1* gene products, a model of seven membrane-spanning helices is predicted for both hCEPT1p and hCPT1p (Figure 10). Chimeric enzyme studies of the *S. cerevisiae CPT1* and *EPT1* gene products have identified a linear region of 218 amino acids sufficient to confer DAG acyl-chain specificity (Hjelmstad et al., 1994). Site-directed mutagenesis of a number of residues within the second predicted DAG binding helix supported the predicted model of hCEPT1p membrane topography (Figure 10): the DAG substrate spans only half of the membrane bilayer, and altering residues predicted to be opposite the catalytic region had no effect on the CPT activity of hCEPT1p. Of note is an amphipathic helix spanning residues 151–168 of hCEPT1p that is also present within the corresponding region of each CDP-alcohol phosphotransferase isolated to date (Dewey et al., 1994; McMaster and Bell, 1997; Williams and McMaster, 1998). The final two aspartate residues of the CDP-alcohol phosphotransferase catalytic motif for each enzyme lie within this amphipathic helix. These two aspartate residues are the catalytic residues within this motif responsible for a predicted nucleophilic attack of the hydroxy group of the hydrophobic diacylglycerol on the phosphoester bond of the water-soluble phosphobase substrate (Williams and McMaster, 1998). We hypothesize that the amphilicity of this helix is required to allow the interfacing of the hydrophilic CDP-choline substrate with the hydrophobic diacylglycerol substrate.

Consistent with our hypothesis that hCPT1 and hCEPT1 functions may not be metabolically distinct was an examination of the EST database, which failed to uncover other mammalian cDNAs with similarity to hCPT1 or hCEPT1. This implies the two cDNAs isolated to date likely comprise the total complement of human CPT- and EPT-encoded activities. This would be similar to the demonstrated gene complement in *S. cerevisiae* (*CPT1* and *EPT1*) (Hjelmstad et al., 1994), and an examination of the completed *C. elegans* database also revealed only two genes coding for enzymes with similarity to hCPT1 and hCEPT1 (Stein, 1999). Inconsistent with the hypothesis that hCEPT1 may function in both the CDP-choline and CDP-ethanolamine pathways in mammalian cells is the report of an EPT-specific enzyme purified to near homogeneity from bovine liver (Mancini et al., 1999). However, our results have shown that when assayed in the presence of Mn^{2+}, hCEPT1 is primarily an EPT (Figure 13), which could explain the results observed using the purified EPT, which was assayed in the presence of Mn^{2+}. It will be interesting to determine if the purified EPT is indeed coded for by the bovine CEPT1 gene, but confirmation awaits peptide sequencing of the purified protein.

A search for regulatory domains within hCEPT1p did not reveal any obvious motifs; however, several interesting regions of similarity were noted. Residues 18–34 of hCEPT1p align with residues 193–209 of argininosuccinate lyase. A previous study observed that CPT became rate-limiting for PC synthesis in livers of fasted hamsters (O and Choy, 1993). The inhibitor was purified and identified as argininosuccinate. In addition, residues 252–277 of hCEPT1p align with residues 89–114 of the platelet-activating factor receptor (Leslie, 1997; Snyder, 1997; Venable et al., 1993). The *S. cerevisiae* Cpt1p and Ept1p enzymes require activation by their products, PC and PC/PE

respectively, in amounts indicative of a precise phospholipid-binding site within the enzymes (Hjelmstad et al., 1994; McMaster et al., 1996). In addition, work in our laboratory has demonstrated that hCEPT1 also requires PC for enzyme activity *in vitro* in a mixed micelle assay that uses sodium cholate. It is not known whether hCPT1 requires phospholipids for activity as no mixed micelle assay has been developed. Therefore this region might be capable of binding phospholipids. The active site of hCEPT1, but not of hCPT1, was also found to have identity with a portion of the PE-binding protein, which has been isolated from a number of sources. This is interesting in light of the ability of hCEPT1, but not hCPT1, to synthesize PE.

B. Metabolic partitioning of pathways for PC and PE synthesis in *S. cerevisiae*

The metabolic partitioning of the Kennedy pathways for the synthesis of PC and PE has been controversial. *In vitro* analysis of purified enzymes and cloned gene products has demonstrated that several enzymes of the Kennedy pathway possess the capacity to use both ethanolamine- and choline-pathway components. Thus, it has been hypothesized that the two metabolic pathways may contain the same enzyme components, or that dual substrate specificity of some of the enzymes may at a minimum result in overlap between enzymes that possess substrate specificity and those that are promiscuous in their use of substrates. Most notable among these *in vitro* results are the ability of some mammalian kinases to phosphorylate both choline and ethanolamine (Kent, 1995; Porter and Kent, 1990), and the ability of some phosphotransferases to use both CDP-choline and CDP-ethanolamine for the synthesis of PC and PE (Hjelmstad et al., 1994). Recent *in vivo* observations have resulted in the conclusion that the ability of the CDP-ethanolamine pathway enzymes to use CDP-choline is restricted to the *in vitro* situation, as

radiolabelling experiments in yeast carrying inactivated genes for various enzymes within the Kennedy pathways revealed that there is strict metabolic pathway partitioning. However, this observation is challenged by the cloning of the human complement of CPT and EPT enzymes described in this work. The human CPT1 product specifically uses CDP-choline as its phosphobase donor *in vitro* and metabolic experiments demonstrated that hCPT1 reconstituted only PC synthesis *in vivo* in yeast devoid of their endogenous CPT and EPT activities. However, the human CEPT1 product could use both CDP-choline and CDP-ethanolamine *in vitro* and was capable of reconstituting the synthesis of both PC and PE in yeast *in vivo*. Our current study supports and extends this observation. We have found a yeast strain in which endogenous yeast EPT1-derived CPT activity can contribute to 60% of net Kennedy pathway-derived PC synthesis and over-expression of *EPT1* reconstituted PC synthesis to wild-type levels. The precise mechanisms that allow this apparent overlap in substrate specificity by *EPT1* remain to be identified, but it is clear that the Kennedy pathways are not strictly partitioned and can significantly overlap at the ultimate step in the synthesis of PC and PE.

C. PC synthesis and SEC14-mediated vesicle trafficking

SEC14 codes for an essential yeast PC/PI transfer protein. Previous experimentation had demonstrated that inactivation of the *CPT1* gene, and the other enzymes within the Kennedy pathway for PC synthesis, allowed cells to survive in the absence of a functional *SEC14* gene product, but that inactivation of the *EKI1* and *EPT1* genes for the synthesis of PE could not bypass the cellular requirement for *SEC14*. The current paradigm whereby *SEC14* serves to mediate Golgi DAG levels to maintain secretory competence is not consistent with the ability of inactivation of *CPT1*, but not *EPT1*, to

bypass the essentiality of *SEC14*, as both *CPT1* and *EPT1* gene products directly consume DAG. However, the present study demonstrated that the rate of endogenous PC synthesis, and hence DAG consumption, directly correlated with the level of *SEC14*-dependent invertase secretion. Thus, these data predict that the rate of endogenous DAG consumption by *EPT1* is normally sufficiently low so as not to contribute significantly to DAG metabolism. Our cloning of the human CPT1 and CEPT1 gene products allowed us to test the role of each of these enzymes in their ability to interact with the *SEC14* secretory apparatus. We observed that expression of human CEPT1, but not CPT1, was able to mimic endogenous yeast *CPT1* and prevent cell growth in the absence of a functional *SEC14* gene product in CTY434 yeast (*sec14*^{ts} *cpt1*). We also noted that expression of human CPT1 restored PC synthesis to levels 70% of that provided by expression of human CEPT1. Thus, a positive correlation exists between endogenous DAG consumption and restoration of *SEC14*-dependent vesicle trafficking to levels required for cell viability.

Consistent with the variations in endogenous DAG consumption affecting *SEC14*-mediated cell growth and secretory capacity was our demonstration of the ability of exogenous di8:0 DAG to rescue *sec14*^{ts} mediated cell death. This observation was complemented by the first assessment of the effects of di8:0 DAG on DAG pool sizes and lipid metabolism in yeast. Di8:0 DAG entered yeast cells and accumulated to levels 20-40 fold higher than those of endogenous long-chain DAG. In addition, di8:0 DAG did not alter the rate of synthesis of PC, PE, PI, PS, PA, CDP-DAG, phosphatidylglycerol, or cardiolipin.

An interesting observation was that hCEPT1, but not hCPT1, was capable of

complementing yeast Cpt1p in mediating *SEC14*-dependent cell growth (Figure 44). This could be explained in several ways. Although hCPT1 was able to reconstitute the CDP-choline pathway in *S. cerevisiae* HJ091 (*cpt1::leu2 ept1*), it synthesized PC at approximately 70% of the level of hCEPT1. There may exist a threshold of PC synthesis required to complement yeast Cpt1p, and the level of PC synthesized via hCPT1 is simply not enough for complementation. Another explanation may be the subcellular location of hCEPT1 versus hCPT1, which are localized to the ER/nuclear membrane and Golgi apparatus, respectively.

The hypothesis that *SEC14* impacts on the regulation of Golgi DAG pool sizes is consistent with the observation that PC-bound *SEC14* protein inhibits PC synthesis by inhibition of CTP:phosphocholine cytidylyltransferase (*PCT1*), the rate-limiting step in PC synthesis. Inhibition of Pct1p activity decreases the availability of CDP-choline for use by Cpt1p and Ept1p for PC synthesis and DAG consumption. Inactivation of the yeast phospholipase D gene (*SPO14/PLD1*) in each of the *sec14*-bypass mutants reimposes the requirement for a functional *SEC14* gene product. If the DAG consumption hypothesis is correct, then the PA generated by *SPO14*-derived activity would be predicted to be metabolized to DAG; however, the PA phosphatase genes required to directly test this hypothesis have yet to be identified in yeast.

An interesting observation during the *SPO14*-inactivation analysis revolved around the *SAC1* gene. The *SAC1* gene codes for a PI-4-P phosphatase and was one of the original *sec14*-bypass suppressors. Inactivation of *SAC1* results in a 5-fold increase in PI-4-P levels and hence it was proposed that PI-4-P levels directly correlated with the ability of *SEC14* to function during vesicle trafficking. However, inactivation of *SPO14* in the

sec14^{ts} *sac1* yeast reverses the bypass effect of *sac1* and results in decreased secretion and eventual cell death, even though PI-4-P levels remained high. Thus, the role of PI-4-P in mediating *SEC14* function is still unclear. In one of these studies it was noted that *sac1* mutants display a dramatic increase in flux through the CDP-choline pathway for PC synthesis. Inactivation of *SPO14* in *sac1* yeast restored PC synthesis to normal rates, indicating that *SPO14* generated products directly affecting PC synthesis. The most likely explanation for these results is that the PA generated by PLD hydrolysis is normally converted to DAG for consumption by *CPT1*- and *EPT1*-derived phosphotransferase activities.

The challenge in analyzing alterations in lipid metabolism in the face of *SEC14* dysfunction, and the accompanying bypass suppressors, is addressing which lipids play a direct role in modulating protein secretion and cell growth. The data presented in this study include correlations of endogenous DAG consumption with invertase secretion indices and cell viability, and exogenous DAG administration affecting *SEC14*-mediated cell death. In addition, exogenous DAG did not affect the metabolism of any phospholipid tested, nor was it converted to di8:0 phosphatidic acid, implying that DAG itself was likely the mediator allowing *SEC14*-dependent alterations in protein secretion and cell growth. The most obvious steps in the elucidation of the precise mechanisms of *SEC14*-mediated protein secretion will be an assessment of whether the lipids themselves promote vesicle fusion, or whether there are protein targets for lipid activation or inhibition, and an assessment of how these impact on *SEC14*-mediated vesicle trafficking.

VI. Future directions

The cloning and initial characterization of hCEPT1 and hCPT1 presented in this study have provided us with a wealth of preliminary data with which to further characterize PC and PE biosynthesis in mammalian cells. There are a number of details requiring immediate attention in light of these findings. One of the first objectives would be to develop antibodies capable of differentiating between hCEPT1p and hCPT1p, which has proven difficult due to the high level of identity between the two. The studies presented here have been performed with cells overexpressing either hCEPT1 or hCPT1, and while this is adequate for initial studies, the endogenous proteins must also be characterized. Antibodies generated towards hCEPT1 and hCPT1 would be useful in determining the subcellular localization of the endogenous proteins, as well as determining their tissue and cell type specificity, which would give us insight as to the function of the redundancy of the pathways.

Another crucial experiment is to determine the *de novo* substrate specificity of hCEPT1p and hCPT1p, which could be accomplished *via* deuterium labelling of choline and ethanolamine for their metabolic incorporation into PC and PE. Subsequent mass spectrometry analysis would reveal the molecular species of PC synthesized and therefore give some insight into the DAG specificity of both hCEPT1 and hCPT1. These experiments would ultimately be performed in cells where either hCEPT1 or hCPT1 can be expressed (such as *S.cerevisiae* HJ091) or in cells which specifically express one protein or the other, which could be determined using specific antibodies.

One of the major directions to take with this research would be to determine why a redundancy for PC biosynthesis exists, as seen by the ability of both hCEPT1 and hCPT1

to synthesize PC. It would be interesting to determine the consequences of a loss of function for either protein, either through antisense technology or *via* whole animal or tissue-specific knockout mice. Tissue-specific expression of hCEPT1 and hCPT1 could aid in the determination of their roles in PC plasmalogen synthesis in the heart, or PAF synthesis in macrophages, for example.

Another interesting area to pursue is the colocalization of hCEPT1p with CTα at the nuclear membrane, and whether the two proteins physically interact *in vivo*. This could be accomplished *via* immunoprecipitation experiments or a 2-hybrid analysis.

This work has not only revealed specific findings with regards to PC and PE biosynthesis in mammalian cells, but has also given us some general ideas and generated more questions concerning global phospholipid synthesis and trafficking of lipids intracellularly. One of the main questions arising from this work has been whether the site of turnover of phospholipids (ie: via the action of phospholipases) is the same as the site of synthesis. To resolve this, an in-depth characterization of the various phospholipid-synthesizing enzymes and the turnover of their products would need to be initiated. If the site of synthesis were different than the site of turnover, this would lead to questions regarding the involvement of phospholipids in trafficking events. Although a link has been established between phospholipid synthesis and vesicle-trafficking events, it remains to be seen how enzymes involved in phospholipid biosynthesis exert effects on intracellular trafficking events. One possibility is that the integral membrane proteins involved in phospholipid biosynthesis actually work to modify the membranes destined for vesicle formation. Alternatively, the enzymes may interact with other proteins, which together can influence vesicle formation and subsequent trafficking events.

VII. References

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